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# ADVANCES IN PEST CONTROL RESEARCH

Volume I

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# ADVANCES IN PEST CONTROL RESEARCH

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VOLUME I

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# Preface

Pests—organisms that compete with man for his food supply, damage his possessions, and attack his person—are of vital concern to all mankind. Included in this category are some thousands of economically destructive species of plants and animals, variously classified as viruses, bacteria, fungi, weeds, protozoa, nematodes and other helminths, molluscs, insects and other arthropods, fish, birds, and rodents. The accumulated attacks of all of these pest organisms cost the human race incalculable damage in direct loss of vital foodstuffs, in enormous expenditures for pest control activities, and in pestilence and disability.

Pest control in its broadest sense is a continuous warfare against man's competitors, carried out on countless fronts with operations ranging in scope from those of the housewife and home gardener to the global efforts of the Food and Agriculture Organization and the World Health Organization of the United Nations.

Throughout the centuries, pests have demonstrated amazing plasticity in their abilities to circumvent man's best efforts to control them, and presently it is abundantly clear that research in pest control not only is essential to ensure human enjoyment of the fruits of the earth but also is capable of yielding unsuspected dividends in terms of an expanding population with abundance for all.

Pest control research currently occupies the attention of a significant portion of the world's scientific manpower, at work in university, governmental, and industrial laboratories, and in field experimentation the world over; increasing the basic knowledge of our animal and plant competitors and developing and applying more suitable and effective measures for their control. Although these research endeavors are often carried out under the various classical disciplines of plant pathology, entomology, economic botany and zoology, it is apparent that they have much in common. This has been recognized by the employment of such terms as plant protection or Pflanzenschutz research. Furthermore, progress in pest control research is directly dependent upon the ancillary disciplines of chemistry, physics, ecology, and pharmacology. It is therefore

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to this broad field of endeavor that *Advances in Pest Control Research* is addressed.

The subject matter will be selected from recent significant research trends related to all phases of pest control, with emphasis on the fundamental aspects. As in other "Advances," the writers will be specialists in the various fields who will be urged to contribute not only comprehensive reviews but also critical evaluations of new concepts and developments. It is hoped that by the encouragement of the presentation of personal viewpoints, *Advances in Pest Control Research* will become a stimulating vehicle for the fostering of new lines of research as well as a continuing reference work for the research worker, the teacher, and the student. Advice and counsel leading to the best approaches to these objectives are earnestly solicited.

R. L. METCALF

*Riverside, California*
*June, 1957*

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# CONTROL OF HEALTH HAZARDS ASSOCIATED WITH THE USE OF PESTICIDES

By J. M. BARNES, *Carshalton, Surrey, England*

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## I. Introduction

A general survey of the health hazards associated with the use of pesticides was made during 1952 under the auspices of the World Health Organization and the findings published in 1953 together with a bibliography (7). The present article attempts to bring that review up to date. There can be no doubt of the continued concern felt by individuals and public bodies about the possible risks to health that may be incurred by the general population as a result of the ever increasing use of chemicals to control pests. The World Health Organization remains fully aware of the problem and continues to organize active studies in this particular field.* The Organization also makes use of many pesticides in the disease control programs which it supports. In general it can be said that the position has changed little since 1952. There is no doubt that those who are in close contact with certain pesticides during their manufacture or application run real risks of poisoning. On the other hand, at the present time there is no evidence to suggest that the general population, who may benefit considerably from the use of these materials, is adversely affected by the toxic properties possessed by the pesticides.

The use of pesticides is likely to continue and to increase. Despite food surpluses in a few areas of the world, pesticides will continue to be used to increase yields, improve quality, and facilitate storage. In public health they will be introduced to control other diseases, when the major causes of death such as malaria have been reduced or eradicated. Diseases hitherto considered to be of lesser importance will then assume a new significance. Finally the reaction of the insects themselves to this attack by chemicals is such as to pose fresh challenges to the synthetic chemist on the one hand and the practical entomologist on the other (88). The resistance of insects to insecticides has been recognized by the World Health Organization as one of the most serious challenges which faces it (138). New chemicals or new uses for the more toxic materials may all find a place in meeting this challenge which is, to some extent, a race against time.

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* *World Health Organization, Tech. Rep. Ser. No. 114*, WHO, Geneva, 1956.

During the past several years there have not been a great many new insecticides introduced into widespread use. Some of those in use today must almost approach the limits of effectiveness from the point of view of their inherent toxicity to insects. There has been a welcome trend toward the development and introduction of organophosphorus insecticides less toxic to mammals. On the whole, however, the health hazard associated with the use of most modern pesticides is not great enough to provide an urgent stimulus to the production of materials safe to handle. It seems reasonable to predict that there may be a slow turnover of pesticides in common use over the next 10–20 years, with the development of some which may be extremely effective for limited purposes. There seems to be a need for efficient nontoxic fungicides and developments in the field of antibiotics for this purpose might be expected here.

This trend of gradual change may disturb those who fear for the public health on the grounds of an exposure to an ever increasing number of chemical substances. On the other hand, the constant changing of the nature of the chemicals to which the population is exposed, does reduce such risks as may be dependent on a lifetime's exposure to small doses of any particular material.

It is impossible to collect reliable data to show whether the general incidence of true poisoning by pesticides is going up or down. While there seems good evidence that the use of pesticides is increasing steadily all over the world, a survey of the medical literature in the major European languages provides no evidence of a corresponding increase in the reports of poisoning, either real or suspected. That pesticides in general continue to enjoy a reputation for toxicity there is little doubt, but this reputation rests to some extent upon the reactions seen among those who misuse the materials. Accidents, suicides, or even homicides may together account for more deaths than have arisen from legitimate but careless usage. At least that is definitely the case with parathion (103).

In spite of a general awareness of the real and potential hazards arising from the use of pesticides there persists this underlying fear in many quarters that some danger exists. This fear, like many others, is occasioned to some extent by ignorance. While there is little that can be done to dispel ignorance among those who do not really want to find out, there is still much more to be discovered about the toxic properties of pesticides before it will be possible to give a really informed opinion of likely hazards that may arise from any sort of exposure to them. In the following pages attention will be drawn to such information as already exists and indications will be given of the directions in which more knowledge should be sought. There is every likelihood that an increasing amount of scientific information will become available on the toxic properties of pesticides both to pests and toward mammals. This will form a basis for understanding the real hazards associated with contact with pesticides.

It is much more difficult to assemble data from which it might be possible to draw a conclusion that no hazard existed. Cases of obvious poisoning draw attention to themselves and investigations will follow. On the other hand, it is difficult to feel enthusiastic about undertaking an investigation to show whether or not contact with a given insecticide is associated with any unusual incidence of disease or disability. Yet proper reassurances about the harmlessness of pesticides, or any other new chemical with which human contact is widespread, must be based on a proper examination of exposed populations. The work itself is likely to be dull and scientifically unrewarding. It may well be expensive and difficult to organize and carry through. On whom does the responsibility for such an investigation rest? It almost certainly falls on the public health authorities at either a national or international level. Such detailed investigations may be undertaken one day under national or international sponsorship, but meanwhile it seems reasonable to assume that in a world already made aware of a potential risk to health, no very obvious manifestations of pesticide toxicity are likely to escape notice for long. The use of pesticides continues to bring much benefit to the human race, and in the absence of positive indications to the contrary their use is likely to continue to be officially encouraged. At the same time articles continue to appear which carry the necessary warnings and appeals for care in handling and using these materials (32,55,56,71,83,104–107,120).

## II. Toxic Properties of Pesticides

The understanding of any health hazard that may arise from contact with a chemical substance demands knowledge both of the toxic properties of the substance and the degree and manner in which exposure has taken place. In this section an outline will be given of some recent developments in our knowledge of the toxic properties of some important pesticides.

The ideal pesticide is one that is toxic to the pest and harmless to other forms of life when used in a manner that will effectively control the pest. As a preliminary to exploring possibilities of this kind a review of the information on the comparative toxicity of certain halogenated hydrocarbon types of insecticides, to both insects and mammals, was undertaken (136). The conclusion reached was that at comparable tissue concentrations insects and mammals probably respond in a similar way to a diverse selection of the members of this particular group of insecticides. Despite obvious structural differences and very important physiological distinctions it seems probable that the basic cellular metabolic processes in insects and mammals are essentially the same. Therefore any substance that is toxic by virtue of its power to interfere at some stage in cell respiration, for example, will be equally toxic to insects and mammals provided that access to the site of action is as easy in the one as in the other. This is obviously not true, for example, in the case of a substance able to penetrate with equal facility the integument of both insects and man. The ratio of body size to body surface is so different that the vital centers of the one lie a few microns, and of the other several centimeters, below the surface. Thus DDT is a safe and effective insecticide because under conditions of normal use it will rapidly gain access to the vital centers of an exposed insect in a concentration great enough to kill it. A similar concentration could not be built up in the vital centers of a man exposed at the same time. Man comes into danger only when he handles insecticides in concentrations that may be a thousand or more times those needed to kill insects. These considerations probably apply to the whole family of the chlorinated hydrocarbon insecticides. They are insoluble in water and probably only slowly absorbed either from the gut or through the skin. The really dangerous members of this family of insecticides are the volatile ones such as methyl bromide where exposure is by inhalation. This is equivalent to a direct introduction into the blood stream. Other members of this group may be given intravenously under experimental conditions. The approximate $LD_{50}$ to rats of a number of common insecticides given by this route are shown in Table I (8). It will be seen that under these conditions lindane and methoxychlor, for example, appear to be relatively much more toxic than when given by mouth. An explanation of this may be that the mammalian organism can metabolize these compounds comparatively rapidly and turn them into harmless breakdown products (130). This would explain why they are less toxic when absorbed slowly from the gut or through the skin. Now the basic toxic mechanism of insecticides of this type may be similar in both insects and mammals in that they have a similar effect on sensitive sites of action, but other factors associated with the more gross physiological and biochemical differences may come into play. These may modify the toxicity of any particular compound with re-

TABLE I

The Approximate Lethal Dose of Certain Insecticides When Given Intravenously to Albino Rats in Small Volumes (0.05–0.2 ml.) of Solutions in Either Tween 80 or Acetone

| Insecticide | Dose, mg./kg. | Interval between injection and death, min. |
|---|---|---|
| DDT | 75 | 60–90 |
| Methoxychlor | 75 | 30 |
| TDE | 250 | 3–5 |
| Lindane | 5 | 30 |
| Aldrin | 18 | 40 |
| Dieldrin | 8 | 15 |
| Endrin | 1.5 | 20 |
| Allethrin | 18 | 20 |

spect to either the insect or the mammal. If the mammal has enzymes that can rapidly detoxify lindane or methoxychlor, the insect may owe its relative sensitivity to these insecticides to a lack of any such enzymes. Clearly any enzyme capable of detoxifying an insecticide was originally designed for another purpose, and its presence in the one species rather than the other will be connected with some quite independent metabolic process. This important question of metabolism of toxic substances will be discussed below in connection with the organophosphorus insecticides.

There has been little new work on the toxicity of DDT and lindane toward mammals. The general toxicity of aldrin, dieldrin, endrin, and isodrin has been recorded (122), and papers have appeared on the mode of action of aldrin and dieldrin (48,49). Aldrin and dieldrin both increase the excitability of the nervous system and will produce convulsions in the anesthetized preparations. This effect is reversed by barbiturates (pentobarbitone), but a further dose of insecticide will again overcome the action of the sedative. Aldrin, but not dieldrin, is said to have a peripheral action in sensitizing the heart and salivary gland to parasympathetic stimulation. The action of acetylcholine is increased in poisoned animals, but no effect of aldrin or dieldrin on cholinesterase could be detected. Since there is some evidence that aldrin is converted to dieldrin in the body (69) it is not clear why there is a difference in their activities on the nervous system.

In general, the effects of aldrin and dieldrin are similar to those of DDT and BHC in that they disturb the action of the central nervous system. As for many other cerebral irritants and convulsive agents, the barbiturates are effective antidotes. Nothing is yet known about the biochemical or physiological mechanism which lies behind the ability of this group of insecticides to produce convulsions. In neither acute nor chronic toxicity tests have the survivors shown any evidence of permanent neurological damage. There has been a number of reports claiming to demonstrate histological damage in animals poisoned with DDT and BHC, and one paper also describes changes in the brain (76). The significance of such histological changes cannot be assessed very highly as an indication of neurone damage, otherwise more permanent functional aberrations would probably have been observed in surviving animals. Some deviations from normal have recently been observed in the gait of rats given doses of DDT below those producing any obvious toxic effect (75). The experiments were originally designed as behavior studies on rats on diets containing 100, 200, 400, and 800 p.p.m. DDT. No change in their ability to learn was found but during the tests it was noted that the rats on higher levels of DDT walked with a shorter, wider gait than the normal ones. When accurate measurements were made, it was found that on the lowest level (100 p.p.m.) the gait was longer and narrower than normal, suggesting that at high doses there was an abnormal response, but at lower levels an exaggerated normal response. The mechanism of this reaction is being studied.

In some of the early reports, the excessive toxicity of chlordane vapor has now been shown to have been due to contamination by an impurity—hexachlorocyclopentadiene (67). This compound, although not very toxic by mouth is very poisonous when inhaled (123). The presence in small quantities of highly toxic impurities also explained some anomalous findings with aldrin (6).

The observation that DDD brings about a striking necrosis of the adrenal cortex of the dog has been repeatedly confirmed. No evidence could be obtained that the rat was similarly affected (116). In the hope that DDD might have a similar action on the human adrenal a single case of Cushing's syndrome (due to adrenal cortical hypertrophy) was given DDD in doses approaching the toxic level but without effect (114). The chemical characteristics of compounds possessing this particular toxic action have been further studied (81).

From existing experimental information it would appear that these chlorinated hydrocarbon groups of insecticides cause serious but temporary disturbances of function in the central nervous system, but that the mammal has the capacity for metabolizing many of them fairly rapidly to harmless breakdown products. They show differing tendencies to be laid down in body fat, but no specific pathological effect has yet been shown to result from this other than mobilization during starvation.

The toxicity of the organophosphorus compounds has always been closely linked with their ability to inhibit cholinesterase. While this action undoubtedly contributes greatly to the general picture of intoxication produced by these compounds, the exact way the animal is killed is not clear. The major action of these anticholinesterases is an interference with the passage of impulses down the nerves supplying the muscles of respiration (16). This is believed to be a central action but there is no positive evidence that this central action is directly linked with the inhibition of cholinesterase in the brain. It has been shown that TEPP can interfere with the passage of impulses down the somatic nerves even when the cholinesterase in those nerves is by no means completely inhibited (97). Experiments on insects poisoned with DFP suggest that it may not be the action of this compound on cholinesterase that is responsible for the death of the insect (134). The observation that one organophosphorus compound developed for use as an insecticide could produce a serious nervous lesion in man led to the use of chickens in testing further candidate compounds of this type (9). No new insecticides have so far been shown to possess this undesirable toxic property. The general trend has been toward the development of compounds much less toxic to mammals. In Table II are listed some $LD_{50}$ data for rats of some of these new insecticides. This may be compared with the figures of 2 and 10 mg./kg. approximately for the $LD_{50}$ of TEPP and parathion.

TABLE II

The Approximate $LD_{50}$ for Rats of a Single Oral Dose of Some Recently Developed Organophosphorus Insecticides

| Compound | $LD_{50}$, mg./kg. | Reference |
|---|---|---|
| *O,O*-Diethyl *O*-(2-isopropyl-4-methylpyrimidyl-6) phosphorothionate (diazinon) | 100–150 | (14) |
| *O,O*-Dimethyl 1-hydroxy-2-trichloroethyl phosphonate (Dipterex) | 450 | (28) |
| *O,O*-Dimethyl *O*-3-chloro-4-nitrophenyl phosphorothionate (Chlorthion) | 625 | (78) |
|  | 1500 | (29) |
| *O,O*-Dimethyl *S*-(1,2-dicarboethoxyethyl) phosphorodithioate (malathion) | 1400 | (29,51) |
| Tetra propyl dithionopyrophosphate | 1450 | (29) |

If it be assumed that the basic mechanism by which these compounds kill insects and mammals is the same, how is it possible that a compound can still be effective as an insecticide although it is several hundred times less toxic to mammals than related insecticides? Several considerations enter the picture and they are rarely discussed in the toxicity reports of these new compounds. If it is assumed that the toxic action of the organophosphorus compounds is based on their capacity to inhibit cholinesterase, then this, in turn, depends on a number of factors. The more stable a compound the less effective it is as an inhibitor of cholinesterase. The P=S compounds are very poor inhibitors and must first be oxidized to the P=O analogue. Very little is known about the metabolic processes responsible for this change. It takes place in the liver of mammals (23), but the process may differ in the insect. Inhibition of cholinesterase is not irreversible and the speed with which activity returns depends upon the nature of the alkyl groups which are attached to the phosphorus atom of the inhibitor (2). Where these are methoxy, reversal is rapid in the early stages, but if inhibition is allowed to persist by the continued presence of the inhibitor it may pass into a very irreversible stage (17). Inhibition by ethoxy phosphates is always more slowly reversible but it takes much longer to pass into the truly irreversible stage. Isopropoxy compounds produce an initial inhibition that is slowly reversible which rapidly passes into an irreversible state. However, not only does speed of reactivation depend upon the character of the inhibitor, it also depends on the character of the enzyme (22). Finally the capacity of the organophosphorus compound to act as an inhibitor at all depends upon its remaining intact until it reaches cholinesterase. The presence in the insect or mammalian tissues of enzymes able to hydrolyze the inhibitor without themselves being inhibited might significantly reduce the toxicity of any particular compound. The toxicity of a new organophosphorus compound will therefore depend on a number of factors each of which may play a relatively different role in insect and mammal and so determine whether the ratio of its toxicity toward the one and the other is high or low. Such factors are: (*a*) the conversion of the original insecticide material to an active inhibitor of cholinesterase, usually by oxidation; (*b*) the relative affinity of the compound for the different cholinesterases, hence its activity as an inhibitor; (*c*) the speed of reversal of the inhibited enzyme; and (*d*) the hydrolysis of the inhibitor by independent enzyme systems.

These points have been made in the hope that they will form the basis of an understanding of why new insecticides in this group may show striking differences in their relative toxicity to insects and mammals. Of the less toxic compounds listed, three are methoxy phosphates which give a rapidly reversible inhibition of cholinesterase, and four of them are thionophosphates which will require preliminary oxidation before they can act as enzyme inhibitors. Where both are combined as in malathion and Chlorthion it is easy to see that if the rate of oxidation is slow the rate of reversal of inhibition may keep pace with fresh inhibition by newly formed inhibitor. If there also exist esterases capable of hydrolyzing the original compound, still less will be available for the formation of toxic material. It may also explain instances where there is a difference in species sensitivity, and where the likelihood of this becomes considerable so does it become more dangerous to extrapolate from laboratory animals to man. As an example diazinon which is of relatively low toxicity to most mammalian species is much more toxic to birds (8). Fortunately these compounds all inhibit cholinesterase in the course of the intoxication process and for the prevention and diagnosis of suspected poisoning in man determinations of blood cholinesterase activity may be used. Some of the organophosphorus insecticides are used as systemics, i.e., taken up by the plant before they kill the insect that feeds on the plant. Schradan, demeton, and methyl demeton are the best known examples. Schradan and demeton are metabolized in both the insect and mammal before they exert their maximum toxic effect (91,98). The metabolism of the systemics in plants and its relation to the residue problem are discussed later.

In most of the published work on the toxicity of the organophosphorus compounds scant attention is paid to the problems of metabolism and conversion of the original material, the possibilities of isomerization and the presence of impurities. Work with methyl analogue of demeton (Metasystox) (77) has reëmphasized this difficulty. In our laboratory it has been found that dilute solutions were found to increase in toxicity by a factor of 30, and an allegedly pure isomer having the theoretical amount of phosphorus contained two compounds, one soluble in benzene and the other in water (127). Possibilities of this kind may make little difference in the value of an insecticide in the field, but they complicate any study designed to elucidate their precise mode of action.

The nitrated phenols and cresols continue to be used as herbicides and as insecticides for killing locusts. The essential features of the intoxication produced by them are well understood, although the exact biochemical mechanism has not yet been determined. Pentachlorphenol has been shown to have a very similar biochemical action to the nitrated phenols (131), and the clinical history of recent cases of intoxication by pentachlorphenol (see below) supports the idea that its toxic action also is similar to that of the nitrated phenols. Both groups of compounds interfere with the production of the high energy phosphate compounds essential to cell respiration. Presumably in the attempt to synthesize enough ATP the cellular metabolism is stimulated and this excessive activity is responsible for the signs of poisoning. Recovery may be rapid but death, if it occurs, is also sudden and probably comes about when even the excessively active cell metabolism can no longer synthesize amounts of ATP adequate for the proper functioning of the brain and heart.

Of the fungicides in common use the mercurial compounds continue to offer the most serious health hazard. All mercurials are toxic to some degree and this property possibly depends on their capacity for combining with and immobilizing sulfhydryl groups which are so important in many enzyme reactions. What is not yet understood are the factors that determine the capacity of any particular mercury compounds to injure the nervous system and the nature of the injury so produced. While some compounds such as the ethyl and methyl mercury derivatives can produce gross cellular injury to the brain, others, including mercury itself, produce reversible behavior and character changes which may be difficult to detect.

This outline has been given in the hope that it will provide some kind of baseline for understanding the nature of the toxic processes produced by the pesticides in common use. Apart from the mercury compounds and isolated organophosphorus compounds, only one of which is in restricted use as an insecticide, the compounds discussed produce what are essentially biochemical lesions in poisoned animals. Although severe poisoning may be fatal, recovery when it occurs is complete. This is understandable on the basis of what is known about the way in which these substances exert their effects. This is a very important point to bear in mind when considering suggestions made to the effect that use of these materials may be associated with a general threat to the health of the public. The mammalian organism can deal rapidly and effectively with all but a lethal dose and it seems therefore unlikely that it will encounter any special difficulty in dealing with very much smaller doses acquired casually by ordinary human contacts. Continued ingestion of materials that are stored in the tissues presents a special problem to which no answer can yet be given.

## III. Manufacture and Distribution

Few accidents or illnesses have been reported among those exposed to pesticides during manufacture. The primary producers have experience in handling toxic chemicals of all kinds and know how to control hazards of this type. Some of the primary producers have extended their responsibilities by advising and controlling the activities of the firms who formulate their products. Nevertheless it seems probable that the greatest risk must lie in formulating plants which vary greatly in their degree of technical development. A true picture of the health hazards in this section of the pesticide industry could not be obtained without special inquiry, particularly in those areas of the world from which published data and official reports rarely appear. The few accidents that have been reported have occurred during the manufacture of parathion. In one particular factory where such cases had occurred, the environmental control was effected by assaying the cholinesterase inhibitory capacity of the parathion trapped in air samplers. This technique had been developed because of the difficulties of analyzing the impure material in factory atmospheres. It was claimed that an atmosphere was safe provided that the contents of 1 liter did not inhibit the cholinesterase activity of human blood (0.5 ml.) by more than 5% (139). Since pure parathion is itself a very poor inhibitor (26) of cholinesterase *in vitro* this may well have given rise to a false sense of security. Parathion must be estimated chemically and not biochemically by its action on cholinesterase. In this same paper it is suggested that a fall in blood cholinesterase of 6% at the end of the day will eventually be cumulative though in a previous paper it had been suggested that a fall of 5–15% each evening would have been restored to normal overnight (110). This confidence in the significance of small changes in blood cholinesterase values is not shared by all those with experience in this procedure (see below). Other accidents during the manufacture of parathion have been reported (10,109,111), and another illustrates the possibilities of misdiagnosis when the acute abdominal symptoms of parathion poisoning may be mistaken for those of some surgical emergency (96). A single fatal accident due to chlordane has been reported (25). A girl died very soon after spilling a formulated product over her clothes. No accidents in the manufacture of toxaphene have been recorded (89).

Because of the anxiety so often expressed that some unforeseen chronic toxic effects may result from long exposure to pesticides, there has been some interest in watching the health of workers exposed during manufacture. Two reports have appeared on workers exposed to chlordane, aldrin, and dieldrin (3,108). A total of 58 men exposed for periods up to 3 years were examined and found to be free of any evidence of kidney or liver disease or other gross disorders. Unfortunately there are no adequate records of the degree to which these men had been exposed during the course of their work. Investigations of this type are difficult to organize, and any long term follow up may be made impossible both by labor turnover and changes in the nature of the manufactured materials. It would be more satisfactory if some simple chemical or biochemical test could be devised to measure exposure to this type of pesticide. The routine blood cholinesterase determination will check the degree of exposure to organophosphorus insecticides, but there is much less concern about possible chronic effects from these unstable compounds which appear to be rapidly metabolized by the body and excreted.

The chlorinated hydrocarbon insecticides are more stable and less is known about their metabolism; some are known to be stored in the fat. Hayes (58) states that a beginning has been made in assessing exposure to DDT by measuring the DDA content of the urine, but details are not yet available.* A similar approach might be made to the study of workers making related compounds. If a simple method existed for the determination of organic chloride in urine, it might be worth finding out whether an estimation of the excretion of organic chlorides would give some estimate of the degree of exposure. The same procedures would also be valuable in assessing the exposure undergone by workers in the field.

Some control of the health hazards from pesticides may be achieved by the use of suitable formulations for the active ingredient. Much care has been taken to devise formulations that will not be toxic to the crops to which they are applied, and similar considerations might be given to human health. The problem has been discussed (59). The use of an antidusting powder in dry formulations has reduced the hazard to mixers. There seems to be scope for the development of pastes and thick emulsions instead of the more easily spilt liquid concentrates which spread rapidly over the skin and clothes. A claim that certain emulsifiers make liquid preparations of parathion less toxic by skin application has been substantiated by work with demeton (24). However, in this case the protective power of the emulsifier was only seen with the concentrate and not with the diluted solution prepared from it. The onus for devising safer preparations of this type may well eventually fall on the biggest users who have important responsibilities for keeping high standards of safety. Thus if mosquito or fly resistance to DDT and related insecticides makes the use of parathion and compounds of similar toxicity imperative in public health programs, there may be a big potential demand for preparations having the maximum degree of safety when widely used.

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* See a recent paper: W. J. Hayes, W. F. Durham, and C. Cueto, *J. Am. Med. Assoc.*, *162*, 890 (1956).

An important degree of control of health hazards can be achieved by systems of registration and the control of distribution of pesticides. Control is usually based on existing legislation designed to deal with other poisonous substances. Registration may be used to insure that labeling is properly done. This is largely an administrative problem not suitable for discussion here. Many cases of accidental poisoning that are reported have been associated with the improper use of the pesticide. This points to the need to ensure that some control of the distribution of toxic pesticides is achieved, especially in countries where the level of technical development and general education are not well advanced and the population have little or no experience in the handling of dangerous chemicals of any kind.

## IV. User Hazards

The handling of pesticides during their normal use must inevitably entail the greatest risks of poisoning. The pesticides are applied in many different ways from all types of equipment handled by men and women who may differ widely in experience and general intelligence. A pesticide less toxic to man will inevitably find wider uses than an equally effective one for which special precautions of any kind have to be observed before it can be safely handled. If the incidence of poisoning associated with use of any particular material becomes serious, local or national authorities may intervene in an attempt to control its distribution or use. In some instances serious poisoning may arise as much from the misuse as from the correct use of a product. In order to ensure the safe use of toxic pesticides it may be necessary to exercise certain precautions, but those precautions which are recommended should be as few and as simple as possible. In order that only the appropriate precautions should be recommended it is essential to know exactly how the greatest degree of exposure takes place under each particular condition of use.

Clearly the biggest risk of acute poisoning is run by those who handle the pesticide in its concentrated form. The early casualties from parathion were among those who diluted or mixed the concentrates. This situation calls for the design of suitable containers and equipment so that the operation of adding the concentrate—liquid or solid—to the diluting water can be carried out with least risk of raising cloud dusts or of splashing the skin or clothes. Some suggestions for the design of spouts and fillers have been made (72). The value of certain types of formulation in reducing this kind of hazard was discussed above.

To those who apply the diluted preparations, the main routes of ingestion will be through the skin as the result of splashes and drips or through the respiratory tract from airborne particles. Conditions will vary with indoor and outdoor application. The first published studies of conditions in orchards where parathion was applied reported concentrations in the air of 2–15 mg. parathion/m.$^3$ when dilute sprays were used, and concentrations up to 26 mg./m.$^3$ during the mixing of a wettable powder (73). Other workers reported lower average figures, e.g., 0.09, 0.13, and 0.36 mg./m.$^3$ for sprays, and in one case 0.76 mg./m.$^3$ for mixing (11). In later Canadian work using airblast equipment for the application of parathion the aerial concentrations found were 0.33 mg./m.$^3$ for the operators, rising to 16 mg./m.$^3$ during the short periods when mixing took place (13). In observations in certain tobacco fields concentrations up to 1.76 mg./m.$^3$ of parathion were observed (112). In a recent study of the uses of malathion and Chlorthion for mosquito control over open ground it was found that the mean aerial concentration to which the jeep driver was exposed was about 1.6 mg./m.$^3$ In the aerosol cloud 10 yd. from its origin the concentration was 4.7–9.4 mg./m.$^3$ (18). Other data on drifting parathion clouds showed that a concentration not exceeding the safe limit (0.1 mg./m.$^3$) was reached 260 ft. down wind (12). While the measurement of aerial concentration gives a general picture of the hazard, a more accurate determination of the dose inhaled by exposed individuals was obtained in a different way. Absorbent pads were fitted to the intake of respirators which the men wore during the operations. The pads were then analyzed for the quantity of the insecticide which they had retained. By this technique both the material of small particle size that will be directly inhaled into the lungs and the larger droplets that will be held up in the upper respiratory tract are collected and measured. In the case of the orchard workers handling parathion the amounts inhaled based on the analysis of these respirator pads were higher than the intake that had been calculated from the aerial concentrations estimated by cloud sampling. However, the observations on malathion and Chlorthion work in the open ground with drifting clouds gave the opposite result.

In both sets of observations the calculated respiration intake was extremely small, and the dose inhaled estimated to be less than 0.1 mg./kg. per day. These observations, therefore, suggest that during outdoor work a serious degree of exposure does not take place by way of the lungs and respiratory tract. If this is the case, protection by respirators or similar devices would be of little value even when pesticides more toxic than parathion are used. The problem of vapor did not arise in this work. A similar study under indoor conditions does not yet seem to have been made, but it is a matter of some urgency that data on this should be acquired. Indoor spraying is widely used for insect vector control work and DDT and BHC have been so used in enormous quantities during the past ten years. When the need arises for the use of more toxic materials it is essential to known whether respirators must be worn during the operations. Observations made during the use of diazinon and DDT (95) indicated that unless respirators were worn by operators doing indoor spraying, a measurable depression of their blood cholinesterase took place. As diazinon is one of the less toxic of the organophosphorus compounds these findings suggest that the inhalation hazard from indoor spraying may be an important one. There is a need of some data on the exact form in which the sprayed material is present in the atmosphere under the conditions of indoor spraying. Only when this information becomes available will it be possible to recommend the most efficient type of protective equipment.

As the risk from inhalation in outside work seems to be small, considerable importance must be attached to the evidence provided in the same reports (11,18) that an important degree of contamination of the skin takes place under these conditions of use. The technique used to measure this was the placing of pads of a given size made of absorbent material on the clothes and skin at different sites on the worker. The quantity of pesticide taken up by these pads after a period of normal work was measured. From the degree of contamination of these small measured areas it was possible to calculate the total amount that would have fallen on the areas of skin known to have been exposed. This total was calculated to be as much as 6.4–8.9 mg./kg. parathion per day in orchard workers (11). Repeated exposure to a dose of this size would be expected to produce severe poisoning or at least a very profound fall in blood cholinesterase within a few days. In suggesting reasons why such degrees of intoxication were not observed, the authors point out that absorption would take place slowly over the whole period of the working day. Furthermore, at any time the accumulated material on the skin could be washed off by the worker when exercising normal hygienic precautions. Doses of the same order were found in the study of workers using malathion and Chlorthion in the open (18). However, in the case of these particular insecticides such doses would not be great enough to produce any evidence of intoxication. During this study two interesting points were made in connection with the observations on the jeep driver who did the mixing of the concentrates. Of all the operators studied he had the greatest skin contamination while he was working with malathion. However, some 90% of this contamination was on the skin of his hands for the protection of which he used long heavy rubber gauntlets. It was presumed that his technique of removing his gauntlets was unsatisfactory. The possibility that the concentrates penetrated the rubber is not discussed. The second point concerns the same man. It was found that during the later work in which Chlorthion was used the degree to which he contaminated his forearms was much less than during the earlier work with malathion but no change was noticed "in his technique of handling materials while filling tanks, formulating or driving the jeep." These valuable observations seem to indicate quite clearly that skin contamination is the most important route of absorption in field work with pesticides. The same conclusion has been reached by others with experience in the control of hazards to pesticide operators (32).

The value of rubber gloves is limited and full protective clothing is often impracticable so that the emphasis must be placed on the actual removal of skin contamination by washing. In three unpublished reports coming to the writer's attention the extensive use of parathion in the field has been safely carried out in hot countries by teams relying on washing as the sole preventive measure apart from general care in handling concentrates. There might be a place for including washing at fixed and regular intervals as a standard precautionary measure when handling toxic pesticides.

For indoor work the degree of skin contamination is much greater, and Hayes (57), using similar techniques to those described above, calculated that a daily dose of 30 mg./kg. of DDT reached the skin of operatives doing malaria control work with DDT. Doses of this magnitude of materials more toxic than DDT would quickly prove dangerous if allowed to remain on the skin. Hayes found that a cotton shirt would reduce skin contamination from 1200 mg./m.$^2$ down to 5 mg./m.$^2$, but such protection must clearly be limited by the capacity of the fabric to soak up the spray material. Waterproof garments would be unsuitable for prolonged periods of work so that more information is needed on the capacity of cheap porous clothing to protect the skin. It is also important to know how effectively the laundering of such garments will remove adherent pesticide materials. Again repeated regular washing of the skin would undoubtedly reduce the hazard very considerably.

Casualties among the users of parathion have continued to be reported. The special hazards run by mixers have been demonstrated in field surveys of blood cholinesterase activities in those handling parathion (117). One episode involving 12 workers was attributed to their youth and general unsuitability for handling such toxic material (94). A report of a single case in an experienced worker again emphasizes the difficulty in finding out how and why poisoning has taken place (5). Other reports are equally inadequate in providing clues as to how poisoning took place (119,124). A case of apparent hypersensitivity to parathion has been described (38). There is also an account of a single fatality from demeton (50). Although accident reports for 1954 from California list a total of 103 cases of parathion poisoning of which 73 were in field workers, there were no fatalities (15). There is no indication of the severity of these cases. In Great Britain a single case of parathion poisoning occurred in 1955 in a glasshouse operative. Poisoning, though severe, was not fatal. Evidence available at the present time suggests that exposure was the result of some penetration by the parathion concentrate of the gloves which the man was wearing.

Parathion has been very extensively used in the Mediterranean area for the control of olive fly, but so far there have been no reports of casualties among those handling parathion for this purpose. The situation gives no grounds for complacency but does indicate that the hazards are amenable to relatively simple control measures. The introduction of organophosphorus compounds of much lower mammalian toxicity should further reduce the potential danger.

Among the chlorinated hydrocarbon group of insecticides there have been no published reports of serious or fatal poisoning. A single case of poisoning by BHC was observed in a woman helping to dip calves (61). She responded rapidly to treatment. The potential danger of these materials is indicated by a report of several cases of dieldrin poisoning among a team of public health workers. The men were working in remote rural areas away from the simplest hygienic facilities and slept unwashed in their working clothes for up to 2 weeks at a time. No fatalities were recorded but a number of the men had convulsions (54). A number of illnesses have been ascribed to DDT and BHC because they have occurred in men who had previously been exposed to these insecticides during their work (44,62, 80,101,118). All these cases were suffering from types of disease met throughout the general population and for which it is often impossible to ascribe a cause. Poisoning by DDT and BHC produces a definite clinical picture, and there is no evidence yet available which makes the attribution of other diseases to these substances in any way justifiable.

There have been no recent reports of poisoning by dinitroorthocresol (DNOC) or related phenols among those who have been using them for pest control work. On the other hand pentachlorphenol (PCP) which has a similar biochemical effect has been responsible for a number of deaths among those using it as a timber preservative (31,125). The signs of poisoning and course of the illness were similar to those seen in cases of poisoning by DNOC. The material is used in dilute solutions and it must be presumed that few if any precautions were taken to avoid skin contamination by splashes or soaked clothing.

Among the many fungicide preparations in common use, those containing mercury must be considered to present the most serious potential health hazard. Almost all those in common use are skin irritants and care must be exercised when handling concentrates for this reason alone. The alkyl derivatives of mercury are known to produce severe brain lesions in mammals, and a recent reminder of their potentialities in this respect comes from Sweden (65). The hazard to which field operators are exposed when using mercurials as foliar sprays has not been investigated. It seems probable that the sources of exposure will be similar to those shown for operators handling parathion in orchards (11). However, the risks of cumulative poisoning will be much greater in the case of mercury. The situation seems to require careful watching if the use of mercurials for this purpose continues to increase.

Although it is during the application of pesticides that the greatest opportunities for accidental acute poisoning arise, the ever increasing scale on which they are being employed does not seem to have been associated with a proportionate increase in casualties. Accidents due to gross carelessness and ignorance will continue to occur as long as any toxic materials are used. A general awareness of the risks coupled with education and simple training in a few minimal hygienic measures seems to have been enough to check casualties from handling material like parathion. In Great Britain control of use has been carried a stage further by legislation designed to protect the field worker. Certain agricultural chemicals are scheduled into different categories. So far only the organophosphorus insecticides and the nitrated phenol weed killers have been included. According to the category into which they are placed, so does the user of that particular material have to wear or provide certain protective clothing which in turn may depend upon the type of operation to be performed. Facilities for washing must be provided and the worker must obey certain rules. The Act is enforced by inspectors appointed by the Ministry of Agriculture, but with such a widely scattered field of operation the successful working of the Act depends a great deal on the active cooperation by all who are involved in its operation. The manufacturers have, on the whole, cooperated actively in ensuring that proper precautions are used. Whether or not legislation is considered to be a practical or desirable method for control of these particular hazards, some system for getting information on the nature and scale of pesticide usage in any country is a valuable adjunct toward keeping hazards under control. By this means it may be possible to give warnings before accidents occur and draw attention to possible sources either of occupational poisoning or of food contamination arising from the use of pesticides.

## V. Medical Control and Treatment

The ultimate value of the medical histories of those who have experienced a prolonged and heavy exposure to pesticides has been discussed above. It is to be hoped that some concerted effort can be organized for carrying out such surveys on exposed groups in order that evidence may eventually be assembled which will indicate whether or not these substances have any hitherto unsuspected chronic toxic effect on man.

Meanwhile the prevention and treatment of acute or subacute poisoning is an important aspect of the safe use of pesticides.

All the members of the organophosphorus group in use at present act as inhibitors of cholinesterase when ingested by mammals. It is therefore fortunate that the blood of healthy men and women has a relatively high normal activity of both the red cell and the plasma cholinesterase. This means that changes in the activity of these enzymes may be detectable before other signs of poisoning appear.

Further experience in the use of blood cholinesterase determinations for the control or detection of poisoning by the organophosphorus insecticides has led to the development of simpler methods for making these determinations. Limperos and Ranta (87) first described a simple field test in which the change in color of bromthymol blue in a solution of acetylcholine was estimated at a fixed time after adding a drop of blood. They claimed that they could detect a fall of 25% from normal activity. They recommended the use of sealed tubes shaken and held in the axilla of the subject while the reaction proceeded. The method has been widely adopted because it can be done in the field and requires no special apparatus. In one modification the changes of the color of the dye equivalent to differences represented by 12$^1/_2$% of normal cholinesterase activity have been prepared as colored glass disks. The blood-acetylcholine-dye mixtures are prepared in the usual way and they include a control of normal blood. At intervals the control sample is read in the comparator and as soon as this reaches the stage at which the change in the color of the dye is complete (i.e., 100% activity) the other samples are read in the comparator (35). In another modification the change from green to orange is used as an end point, and this is timed (21). The time taken depends on the environmental temperature, but if all the estimations are done at the same temperature they can be compared with one another. The time taken for the change of color produced by blood with a normal, slight, or severely depressed cholinesterase activity can be worked out for different temperatures. These techniques depend on the estimation of whole blood cholinesterase activity. Although they lack the precision of the manometric or potentiometric methods, they are very simple to perform and will be quite adequate to indicate when any serious degree of absorption of insecticide has taken place. Doubtful cases can be reinvestigated by the more accurate techniques.

A considerable amount of data has accumulated on the levels of cholinesterase in normal populations and the range of variations in individuals from time to time (45,46,137). The range is considerable and the day to day variation in an individual may be as much as 20% of the normal mean value. It is not, therefore, possible to detect a case of mild exposure from a single determination. Where supervision at work is adequate, it is probably reasonable to consider anyone as within the normal range unless an initial determination shows that their level of activity was 50% or less of the mean level for that population (45). Individual fluctuations may reach 30% of the mean normal value, but when this is exceeded it is certain that poisoning is taking place (46). An individual with a high normal starting level may show a drop of 20–30% in activity as a result of exposure but his level will still lie within the normal range for that population. He will, however, still not have reached a stage when poisoning would show itself or his sensitivity to poisoning by further doses has significantly increased. However, if exposure were to continue at the same rate the level of such an individual would soon fall below the normal range. It is therefore essential to have a system for regular blood cholinesterase determinations available wherever groups are likely to be exposed to the insecticides continuously for long periods. Although plasma cholinesterase activity is affected by certain diseases and by malnutrition (70), the red cell enzyme appears to be independent of such influences and changes in activity seem only to be related to specific chemical inhibition.

For detecting exposure to parathion the estimations of *p*-nitrophenol in the urine has proved a simple and sensitive test (85,86). In rabbits and man between 40–70% of a single dose of parathion can be recovered as *p*-nitrophenol in the urine. In the human observation the dose of parathion administered was so small that no inhibition of blood cholinesterase was produced (37). It is suggested that blood cholinesterase determination before starting work should be followed by daily determination of *p*-nitrophenol. If the figure for this rises a check should be made on the blood cholinesterase (128).

In cases of acute poisoning by organophosphorus insecticides atropine remains the basic treatment, which must be supplemented by the administration of oxygen or by artificial respiration when respiratory failure occurs. It is important to keep any case that has had a dose of atropine under observation for the next few hours. A single dose of atropine may have a striking temporary benefit which will later wear off and be followed by signs of severe poisoning. This points to the danger of any system which involves the issue of atropine to field workers for self-medication. Feeling the benefits of a dose of atropine, a man might return to work and so continue to increase his exposure. A detailed account of 46 severe cases of parathion and TEPP poisoning showed that, whereas the 23 cases that received continued and adequate treatment recovered, the remaining 23 cases had only been given late or inadequate therapy and all died (43). The possibilities of a more specific therapy was raised by the observation that hydroxylamine might hasten the hydrolysis of the inhibited (phosphorylated) cholinesterase (132). A considerable number of other compounds have been tested and some shown to be much more effective (17,133). However, the problem is not simple and the effectiveness of this reactivation depends on the nature of the inhibitor, the cholinesterase inhibited, and the duration of the inhibition (17,64). The substances used to reverse the inhibition are themselves toxic. Some success in treating mice poisoned with paraoxon has recently been recorded (74). At the present time no recommendation for the use of these substances in the treatment of human cases of poisoning can be made.

Unfortunately there is no test comparable to that of cholinesterase determination which will detect exposure to and absorption of the chlorinated hydrocarbon groups of insecticides. Mention has been made of the observations that a determination of DDA in the urine may be useful in detecting exposure to DDT. As little or nothing is known about the metabolism of the other members of this group of insecticides a specific test for substances that might be excreted in the urine cannot be devised. A nonspecific test for organic chlorides might be valuable if a simple accurate technique could be evolved. The signs of poisoning by these pesticides all point to irritation of the nervous system and usually culminate in convulsions. Whenever they have been tried in cases of accidental poisoning the barbiturates appear to be very effective in controlling these manifestations. There is no evidence about the value of any particular barbiturate but the long acting phenobarbitone is effective in experimental animals (8) and its use would seem to be indicated on general clinical grounds.

A specific test for DNOC in the serum has been described. It is very sensitive and simple to carry out (52). While the test is uncomplicated in man, a warning has been published that circulating pigments such as carotene may interfere in certain animal species (34). The significance of blood levels of different heights has been discussed and dangerous levels suggested (53). Whatever their precise significance, these raised blood levels do indicate that exposure has taken place and the individual should be removed from further contact with the material until his blood level is reduced. Acute poisoning by the nitrated phenols may be accompanied by severe restlessness and barbiturates have been recommended for treatment of this condition (32). However there were theoretical objections to the use of barbiturates based on the supposed similarity of their biochemical action to that of the nitrated phenols. Subsequent experimental work showed that in animals poisoned with DNOC a therapeutic dose of some quick acting barbiturates might be immediately lethal (36). This suggests the need for caution in the use of barbiturates as sedatives or as anesthetics in persons believed to have been exposed to the nitrated phenol group of herbicides.

## VI. Accidental Poisoning

The fact that modern pesticides are toxic is amply illustrated by the histories of accidental poisoning published in the world's medical literature. Although the organophosphorus insecticides and nitrated phenol weed killers have been the cause of a number of deaths among those using them for their proper purpose, among the other group of chlorinated hydrocarbon insecticides fatal cases of poisoning are practically confined to instances of accident or misuse. The whole problem of accidental poisoning by chemicals has been admirably put into a proper perspective (115). The introduction of these new pesticides has not created a new problem but perhaps added another facet to an existing one.

Fashions in methods of committing suicide change, and the popularity of parathion for this purpose in Germany has been attributed to the availability of the material in small ampoules and to early reports that death was rapid and painless. The usual victims of accidental poisoning are children left to play within reach of discarded containers still holding residues of pesticide concentrates. Typical stories of poisoning in this way by parathion (68), chlordane (25), toxaphene (102), and BHC (113) merely illustrate the general problem which is related to the training of parents rather than the properties of pesticides as such. Adults are usually poisoned by using the contents of one bottle in mistake for another (19), or more widespread poisoning occurs when pesticides are put into containers and put on shelves in the rooms in which food is prepared (66). The storage of a small bottle of parathion in a refrigerator in which food for the dinner table was normally put, also led to a fatality (79). The dominant place of accidents in statistics of poisoning is illustrated by the case of chlordane. Although reputedly a toxic insecticide, there have been reports of only a single fatality during manufacture and four indeterminate accounts of poisoning among agricultural workers. However, there have been 3 deaths, 3 cases of severe and 2 of moderate poisoning as a result of accidents quite unconnected with the proper use of the material (4).

Deliberate misuse of these pesticides may take place among people with a high incidence of personal or domestic insect infestation. The most extensive report of this type of incident refers to the misuse of BHC in rural Greece (20).

The suggestion that the poisoning of innocent neighbors and bystanders might result from the widespread use of pesticides has been raised on a number of occasions. As a result of inquiries from an area where DDT and related pesticides were being widely used in cotton growing, the US Public Health Service carried out an investigation to see whether any change in the incidence or type of natural disease could be related to the introduction of DDT and the new insecticides into the agricultural practice of the area in 1947. School attendance records, mortality data, hospital records, and a rapid medical survey of workers in the cotton fields failed to provide any evidence of a change in the incidence of disease that could be related to the introduction of these materials (42). The report represents a considerable effort with no corresponding reward of scientific interest to the inquirer. It illustrates the difficulty that must always face those who set out to try to disprove allegations of a vague and general nature made originally on the basis of personal impressions or merely emotional in origin. The need for vigilance in this respect cannot be overemphasized, but it will be difficult to embark on field surveys of this kind before some evidence has been amassed either from human or animal experimental observations that specific long term toxic effect can be expected from exposure to particular chemical compounds. Manufacturers, formulators, and contract sprayers have the most heavily exposed sections of the population under their charge and it is from sources of this kind that the first evidence of chronic toxic effects will arise just as they have done in the other industrial processes. The deeper the scientific study of the mode of action of the toxic substances is pushed, so will the chances increase of either being able to foresee any such effects or being able to discount the likelihood of their ever arising.

## VII. Residues in Food

The problem raised by the presence of pesticide residues in food has aroused considerable anxiety both among public health authorities and certain individuals. There have been a number of statements implying that communities might be poisoned by the consumption of food containing such residues (121).

The continued lack of positive evidence of harm to the national health in communities where food treated with pesticides is consumed reduces the occasion for alarm or the need for desperate remedies. At the same time reassurance, if it can continue to be given, needs to be based on whatever scientific data can be assembled. This must show in the first place the actual quantities of the pesticides which are being consumed and then the relation that such a dose level may have to those doses known to produce the first signs of poisoning.

There are two major problems which require different methods for their solution. It is first necessary to know what quantities of the different pesticides are present in the food as it is consumed and what proportion of a population eats such food, and in turn what proportion of their diet such treated food may comprise. The solution of this problem requires proper information on the extent of the use of pesticides and facilities for carrying out the appropriate analytical work which will be directed to the sources of contamination indicated by the first part of the inquiry. While much reassurance might be possible were this simple factual data available there is no belittling the scientific labor in analytical laboratories that would be necessary in order to compile and keep up to date such information on pesticide residues in food. The second problem is that of deciding whether or not a given rate of intake of a pesticide or mixture of pesticides is likely to be injurious to human health. The difficulty here lies in the fact that the dosage of pesticides ingested in the food as a result of the normal and recommended use is very much smaller than the dose that may, in either experimental animals or in accidentally poisoned humans, produce positive signs of intoxication. On the other hand, positive evidence that ingestion has taken place may be obtained by suitable experimental observations that are discussed below. The difficulties lie basically in the definition of the term "chronic toxicity." If it can be defined, it is then necessary to devise the experimental means for establishing its nature or excluding its occurrence in any particular case. When the animal experiments have been completed it is still necessary to translate the findings from these in terms which are applicable to man. Experimental facts may not be in dispute, but the interpretation of the findings may be a matter of opinion. The opinion given may depend upon the particular expert(s) called upon to advise. At this point it is perhaps better to discuss the general situation in terms of a number of specific practical problems.

As the most widely used and probably the most extensively studied of the synthetic pesticides DDT continues, by virtue of its persistence, to occupy an important place in the field of pesticide residues. DDT has been found in the fat of 75% of a group of individuals not occupationally exposed to DDT. It also appeared in human milk (82). Others have reported similar observations on human fat (99). The average level of DDT in the fat of 75 people was 5 p.p.m. (82). Levels up to 50 p.p.m. were found in individuals who had had some known exposure to the inscoticide (99). More precise analytical work showed that from 33–90% of the material was DDE, a metaoblite of DDT (92). Further work has been done to check the source of this DDT and an analysis was made both of meals and individual food items used in public eating places in America. Most of the meals which were analyzed contained some DDT and the average total daily intake of DDT which would have resulted from eating 3 such meals daily would amount to 0.184 mg. DDT or a concentration of 0.31 p.p.m. in the whole diet (129). A rat on a diet containing 5–10 p.p.m. DDT stores it at a rate equivalent to 6–14 times that of the intake (84). On the assumption that man stores DDT at the highest rate found for the rat (14 times), the consumption of a diet containing 0.31 p.p.m. DDT would lead to an accumulation of 4.3 p.p.m. in the fat. This is in reasonable agreement with the mean figure (5 p.p.m.) found earlier for the DDT content of normal human fat. Although individuals may come in contact with DDT in other ways than in their food, it is nevertheless conceivable that the greater part of that which they store in their fat has reached them by way of their daily food. Whether or not this should arouse any disquiet is a matter of opinion. There is no evidence that the DDT in the fat is doing harm and its presence there may only be a reflection of its particularly favorable partition coefficient of solubility in lipoid. It has rightly been pointed out that fat is not an inert reservoir but is continually being "turned over" in the body metabolism and this must simultaneously expose enzymes to the action of DDT and DDE (84). If it was felt desirable to reduce the general level of DDT in human fat, this could be achieved either by restricting the use of DDT or by substituting other products equally effective as pesticides but which do not possess this tendency to be stored in the body fat. On the other hand, so little is known about the real toxic mechanism of these substances that it might not necessarily be less harmful. There is no way of deciding whether it is better to metabolize 1 mg. of insecticide A in 24 hr. or the same quantity of insecticide B in 24 days. If the insecticides were not equally efficient so that more had to be used, the question might turn on whether it was safer to metabolize 2 or 3 mg. of A in a short time or 1 mg. B over the longer period. Opinions might well differ unless the precise mode of action of both substances was known. Common sense emphasizes the desirability of reducing as far as possible the ingestion of materials liable to accumulate in fat, but only if there is reasonable evidence that the substance substituted is no more harmful in any other respect. As in all aspects of this problem difficulties arise because the dosages of the pesticides involved are in the range where no toxic effect can be detected by physiological, pharmacological, or biochemical tests. Although it is said that DDT at 5 p.p.m. in the diet of rats will produce liver cell changes (39), the significance of these has been questioned (58).

Staple dietary articles, especially cereal products, may be treated by insecticides during storage and during carriage in international trade. Problems may be presented to communities with limited food supplies for whom the use of pesticides is an alternative to the threat of famine. Similarly, countries such as the United Kingdom relying for 60% of their food from other countries may have no control over the use of pesticides on the crops before they reach them. Investigations here are required on the proper quantities and correct methods of applying different pesticides to ensure effective pest control. The behavior of individual pesticides needs to be studied in order to find out which persist in the greatest quantities in the food up to the time it is actually consumed. Thus a material driven off readily by heat in cooking might be preferable to a less volatile alternative. Other pesticides might react with the crop as has been shown to be the case with methyl bromide. The reaction products in this case seem to be free of demonstrable toxic effect (135). In addition it has been estimated that in the U.K. only 0.5% of the wheat crop is ever treated with methyl bromide (63). On the other hand, ethylene dibromide though absorbed on to the treated grain does not react with its constituents (100).

Clearly there are many aspects to this problem of pesticide residues in foodstuff where the first need is for quantitative and analytical data in order to be able to assess the hazard at a stage before the question of the potential toxicity of the pesticide residues arises at all. If codes of practice could be formulated and agreed upon for the effective control of pests under different conditions then accurate determinations of the residues in foods could be made. When this information was available medical opinion could be sought on the magnitude, if any, of the risk being run as a result of the use of pesticides to control the infestation of stored staple foods such as grain.

Another problem arises from the control of the dacus fly in olive culture. An effective method of control involves repeated spraying of the trees with parathion (41). This leads to an accumulation of the insecticide in the oil within the fruit so that the eggs laid by the fly in the olive do not mature and spoil the fruit. Inevitably some of the parathion remains in the olive when it is harvested and in the oil extracted from the olive. Olive oil is the main source of dietary fat for many communities in the Mediterranean area. There is unfortunately no body of analytical data which definitely establishes the degree of the contamination of the oil by parathion, but figures from 5–10 p.p.m. have been suggested. Whether or not the consumption of olive oil containing this amount of parathion will have any toxic effect could be determined in the first place by an estimation of the blood cholinesterase levels on people consuming the oil. A measurable and significant reduction of blood cholinesterase activity should precede any more toxic effects. Such an experiment would require careful planning in order to allow for the normal fluctuations in blood cholinesterase levels among the total population. On the other hand it is not possible to say whether, if a 10–20% reduction of activity was observed, this represented a threat to health. It does, however, indicate a departure from normal which should be avoided if possible. The sensitivity of man to parathion in the diet is not known. Rats show effects on their blood cholinesterase when they consume 0.02 mg./kg. per day whereas in a pig 1.0 mg./kg. is needed to produce such an effect (33). Olive oil will not comprise more than about one-fourth of the total daily food intake but it may well be taken continuously throughout the year. The sensitivity of humans to schradan has been estimated and a dose of 1 mg./day (0.02 mg./kg.) produces a measurable (25%) depression of cholinesterase but no other toxic response. The same daily dose produces a similar effect in both pigs and rats (33). In the case of the humans given schradan the cholinesterase activity returned to normal after dosage stopped at the rate of about 0.5% per day. A daily intake dose of 200 g. olive oil containing 10 p.p.m. parathion would represent a dose of 2 mg. (0.04 mg./kg.) parathion per day. Although the acute toxicities of parathion and schradan are of the same order, it is not possible to predict whether man would respond like the rat or like the pig.* In

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* In a letter dated February 1957, Dr. Edson writes as follows:

"Studies have been carried out on the anti-cholinesterase effects of prolonged

*Footnote continued on next page.*

neither case is illness likely to result but a fall in blood cholinesterase would be expected were man as sensitive as the rat. Thus the situation is not likely to be one of immediate danger to health, but it does provide opportunities for field study on a residue problem of considerable importance. No satisfactory observations can be made unless there is a reliable analytical method for estimating the parathion in the olive oil. Work is also needed to substantiate the interesting claim that the material in the olive oil which reacts as parathion is not so toxic as the original insecticide (1).

Other less toxic organophosphorus insecticides may prove effective in the controlling of the dacus fly, but as long as the principle of the method of control is not changed the problem of residues in the oil will remain. If a much less effective inhibitor of cholinesterase is used then the oil may not have any effect on the cholinesterase of the person consuming it, but it may now contain a residue of the hydrolyzed product of the inhibitor which has a completely unknown degree of toxicity.

The use of systemic insecticides offers advantages to the practical entomologist, but adds new aspects to the residue problem. The two most widely used materials, schradan and demeton, have now been

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daily administration of small doses of parathion in the female rat, female pig, and groups of human subjects, with the objective of finding out what intake rates of parathion caused a just-detectable depression of cholinesterase activity. Three five-dosage levels were employed, and the minimum duration of the trials was 33 days, the maximum 122 days. It was found that the red cell activity was the most sensitive index of response to daily administration of parathion, and the results showed that the highest ineffective intake rates, i.e., those causing neither toxic effects nor red cell enzyme inhibition were as follows:

| | | |
|---|---|---|
| Female rat | 0.02 mg./kg./day | (approx. 0.2 p.p.m.) |
| Female pig | 1.0 mg./kg./day | (approx. 20 p.p.m.) |
| Adult human | 0.06 mg./kg./day | |

In the human experiments, four subjects ingested 4.8 mg. parathion on 45 days during a period of 63 days of the trial, and weekly whole blood ChE measurements showed no significant depression. A further group of four subjects ingested 7.2 mg. on 30 days in a period of 42 days, and this caused 33% inhibition of mean initial ChE activity. If humans can withstand parathion dosage without detectable response at a daily intake rate of 0.06 mg./kg./day, a 60-kg. human could ingest 3.6 mg. parathion per diem, equivalent to 1 kg. of food containing 3.6 p.p.m. residual parathion. Such intake rates give a very wide margin of safety from those likely to cause any toxic effects or illness attributable to parathion."

extensively investigated. It is clear that the metabolism of demeton in the plant takes the same course as it does in the mammal so that the consumer of treated produce is exposed to no greater hazards than those arising from the quantity of demeton originally present (93). In the case of schradan the insecticidal activity within the treated plant depends upon the residue of unchanged schradan and the metabolic products do not accumulate (60). These careful studies indicate the demands made on the analytical chemist and the biologist to provide scientific data to satisfy a reasonable anxiety that systemic insecticides might in some way become changed into some more toxic materials once they were inside the plant. Further evidence in the case of demeton was obtained by feeding treated fruit in normal and excessive quantities to human volunteers (126). No measurable effect on blood cholinesterase activity could be detected. There was a proposal to use another systemic, dimefox, to control the vectors of swollen shoot disease in cocoa. A sensitive analytical method was developed and it was conclusively shown that under conditions of effective use no residues of insecticide remained in the harvested crop (30). Again a question of toxicity was resolved by proper analytical data indicating that in fact a real problem did not arise. An interesting investigation of the residue hazards from aldrin, demeton, and mercury has also been published showing the application of recommended safety factors to the analytical data (47).

There have been proposals for the use of insecticides systemically for the control of cattle parasites. Aldrin, dieldrin, and lindane injected subcutaneously in oil apparently form a depot from which insecticide is slowly liberated to circulate and kill blood-sucking insects (90). Such techniques, if of value for meat producing or dairy cattle, would clearly require much analytical work on residues before they would be acceptable from a public health point of view.

Of the fungicides, mercury probably forms the biggest problem especially as quantities as small as 0.5 p.p.m. in the diet of rats are said to be injurious (40). Copper is probably harmless; small quantities are essential. Selenium is known to be toxic in small quantities and residues are never likely to be permitted.

The effective control of toxic hazards from residues is the concern of the public health authorities in individual countries. From the situations instanced above it is clear that problems take different forms in different countries. Where food is abundant and public health standards are high the authorities may be justified in insisting on the very minimum of contamination of food by pesticides. In poorer countries with limited sources or stocks of food the preservation of this food may be more important than the hypothetical hazard from the pesticides in the food. Much discussion of this problem has taken place in the USA over the past several years culminating in a published list of tolerances of many pesticides in 1955. These tolerances are confined to fruit and vegetables including potatoes but do not include cereals treated in storage or transit. Other countries may feel inclined to accept these limits knowing them to have been set only after exhaustive inquiry. On the other hand their effectiveness as controls must depend upon the existence of a proper service for the necessary analytical examination of the food, in order to control and check the degree of contamination taking place. Other countries may wish to apply limits to different crops and it is not necessarily reasonable to accept the US limits which are limited to fruit and vegetables as being equally applicable to a cereal staple food. Although the limit of parathion on a variety of fruit and vegetables is set at 1 p.p.m., it would not necessarily follow that the same level would be permissible in olive oil where this forms an important daily food. These high standards set by the US Food and Drug Administration, while they may serve as a valuable yardstick, may not necessarily be immediately applicable to situations in other parts of the world. In the United Kingdom there are not legal permitted limits, but certain unofficial working levels are used. The position in many European countries has been summarized (27). It is apparent that the control of residues is largely achieved by restricting the time of the application of the pesticide in relation to the time of harvest. This is a simple method but obviously not offering a complete control. This cannot be achieved under any system without an analytical service.

In general the problem is to avoid the heavy contamination of staple articles of food and drink. This in turn depends in the first place on a proper education in the use of pesticides so that they are properly applied by methods that ensure that they work efficiently. Proper application should also be correctly related to the time of consumption or ultimate processing of the treated food. An all embracing control of pesticide residues in food may finally rest on legislation and permitted levels backed by an effective service for analyzing food for these residues. On the other hand a very satisfactory control of the hazard can probably be achieved in the first place by education into the proper ways of using pesticides so that both the pests and the hazards are simultaneously controlled.

## References

1. Aiazzi-Mancini, M., and Pepeu, G., *Arch. ital. sci. farmacol.*, Ser. III, *5*, 70 (1955).
2. Aldridge, W. N., and Davison, A. N., *Biochem. J. (London), 55*, 763 (1953).
3. Alvarez, W. C., and Hyman, S., *Arch. Ind. Hyg. and Occupational Med.*, *8*, 480 (1953).
4. AMA Committee on Pesticides, *J. Am. Med. Assoc.*, *158*, 1364 (1955).
5. Annis, J. W., and Williams, J. W., *J. Am. Med. Assoc.*, *152*, 594 (1953).
6. Ball, W. L., Kay, K., and Sinclair, J. W., *Arch. Ind. Hyg. and Occupational Med.*, *7*, 292 (1953).
7. Barnes, J. M., World Health Organization Monograph Series No. 16, 1953.
8. Barnes, J. M., Unpublished observations.
9. Barnes, J. M., and Denz, F. A., *J. Pathol. Bacteriol.*, *65*, 597 (1953).
10. Barsotti, M., Sassi, C., and Del Bono, P., *Med. lavoro.*, *45*, 469 (1954).
11. Batchelor, G. S., and Walker, K. C., *Arch. Ind. Hyg. and Occupational Med.*, *10*, 522 (1954).

11a. Blazquez, J., and Bianchini, C., *Gac. med. Caracas*, *63*, 1 (1956).

12. Braid, P. E., Windish, J. P., and Ross, C. R., *Arch. Ind. Health*, *11*, 403 (1955).
13. Braid, P. E., Windish, J. P., and Ross, C. R., *Arch. Ind. Health*, *11*, 408 (1955).
14. Bruce, R. B., Howard, J. W., and Elsea, J. R., *J. Agr. Food Chem.*, *3*, 1017 (1955).
15. Bureau of Adult Health of the California State Department of Public Health, *Arch. Ind. Health*, *13*, 102 (1956).
16. de Candole, C. A., Douglas, W. W., Lovatt Evans, C., Holmes, R., Spencer, K. E. V., Torrance, R. W., and Wilson, K. M., *Brit. J. Pharmacol.*, *8*, 466 (1953).
17. Childs, A. F., Davies, D. R., Green, A. L., and Rutland, J. P., *Brit. J. Pharmacol.*, *10*, 462 (1955).
18. Culver, D., Caplan, P., and Batchelor, G. S., *Arch. Ind. Health*, *13*, 37 (1956).
19. Dadey, J. L., and Kammer, A. G., *J. Am. Med. Assoc.*, *153*, 723 (1953).
20. Danopoulos, E., Melissinos, K., and Katsas, G., *Arch. Ind. Hyg. and Occupational Med.*, *8*, 582 (1953).
21. Davies, D. R., and Nicholls, J. D., *Brit. Med. J.*, *1955*, *I*, 1373.
22. Davison, A. N., *Biochem. J. (London)*, *60*, 339 (1955).
23. Davison, A. N., *Biochem. J. (London)*, *61*, 203 (1955).
24. Deichman, W. B., Brown, P., and Downing, C., *Science*, *116*, 221 (1952).

25. Derbes, V. J., Dent, J. H., Forrest, W. W., and Johnson, M. F., *J. Am. Med. Assoc.*, *158*, 1367 (1955).
26. Diggle, W. M., and Gage, J. C., *Nature*, *168*, 998 (1951).
27. Dormal, S., *Parasitica* (*Gembloux*), *10*, 60 (1954); *11*, 58, Ier Addenda (1955).
28. DuBois, K. P., and Cotter, G. J., *Arch. Ind. Health*, *11*, 53 (1955).
29. DuBois, K. P., Doull, J., Deroin, J., and Cummings, O. K., *Arch. Ind. Hyg. and Occupational Med.*, *8*, 350 (1953).
30. Dupee, L. S., Heath, D. F., and Otter, I. K. H., *J. Agr. Food Chem.*, *4*, 233 (1956).
31. Edson, E. F., Personal communication.
32. Edson, E. F., *Brit. Med. J.*, *1955*, *I*, 841.
33. Edson, E. F., Internal Rept., Medical Dept., Fison's Pest Control Ltd., Cambridge, England.
34. Edson, E. F., and Fenwick, M. L., *Vet. Record*, *67*, 450 (1955).
35. Edson, E. F., and Fenwick, M. L., *Brit. Med. J.*, *1955*, *I*, 1218.
36. Edson, E. F., and MacLeod Carey, F., *Brit. Med. J.*, *1955*, *II*, 104.
37. von Eicken, S., *Angew. Chem.*, *66*, 551 (1954).
38. Engelien, G. A., *Münch. med. Wochschr.*, *97*, 111 (1955).
39. Fitzhugh, O. G., and Nelson, A. A., *J. Pharmacol. Exptl. Therap.*, *89*, 18 (1947).
40. Fitzhugh, O. G., Nelson, A. A., Laug, E. P., and Kunze, F. M., *Arch. Ind. Hyg. and Occupational Med.*, *2*, 433 (1950).
41. *Food and Agr. Organization U. N.*, Report of the Second Meeting on the Control of the Olive Fly, 1955.
42. Fowler, R. E. L., *J. Agr. Food Chem.*, *1*, 469 (1953).
43. Freeman, G., and Epstein, M. A., *New Engl. J. Med.*, *253*, 266 (1955).
44. Friberg, L., and Martensson, J., *Arch. Ind. Hyg. and Occupational Med.*, *8*, 166 (1953).
45. Fryer, J. H., Steel, R. G. D., and Williams, H. H., *Arch. Ind. Health*, *12*, 406 (1955).
46. Gage, J. C., *Brit. Med. J.*, *1955*, *I*, 1370.
47. van Genderen, H., *Voeding*, *16*, 742 (1955).
48. Gowdey, C. W., Graham, A. R., Seguin, J. J., and Stavraky, G. W., *Can. J. Biochem. and Physiol.*, *32*, 498 (1954).
49. Gowdey, C. W., and Stavraky, G. W., *Can. J. Biochem. and Physiol.*, *33* 272 (1955).
50. Griesshaber, A., *Sudwestdeut. Arztebl.*, *7*, 158 (1952).
51. Hagan, E. C., *Federation Proc.*, *12*, 327 (1953).
52. Harvey, D. G., *Lancet*, *1952*, *I*, 796.
53. Harvey, D. G., Bidstrup, P. L., and Bonnell, J. A. L., *Brit. Med. J.*, *1951*, *II*, 13.
54. Hayes, W. J., Personal communication. Now published, see ref. 11a.
55. Hayes, W. J., *Chem. Specialties Mfrs. Assoc. Proc.*, *1953*.
56. Hayes, W. J., *Public Health Repts.* (*U. S.*), *69*, 893 (1954).
57. Hayes, W. J., World Health Organization Expert Committee on Insecticides Working Paper No. 36, 1955.

58. Hayes, W. J., *Am. J. Public Health, 45,* 478 (1955).
59. Hayes, W. J., and Pearce, G. W., *J. Agr. Food Chem., 1,* 466 (1953).
60. Heath, D. F., Lane, D. W. J., and Park, P. O., *Trans. Roy. Soc. (London), B239,* 191 (1955).
61. Heiberg, O. M., and Wright, H. N., *Arch. Ind. Health, 11,* 457 (1955).
62. Hertel, H., *Deut. Arch. klin. Med., 199,* 256 (1952).
63. Her Majesty's Stationery Office. Toxic Chemicals in Agriculture. Residues in Food. London, 1953.
64. Hobbiger, F., *Brit. J. Pharmacol., 10,* 356 (1955).
65. Hook, O., Lundgren, K. D., and Swensson, A., *Acta Med. Scand., 150,* 131 (1954).
66. Hsieh, H. C., *Arch. Ind. Hyg. and Occupational Med., 10,* 344 (1954).
67. Ingle, L., *Science, 118,* 213 (1953).
68. Johnston, J. M., *J. Pediat., 42,* 286 (1953).
69. Jolly, D. W., Personal communication.
70. Kaufman, K., *Ann. Internal Med., 41,* 533 (1954).
71. Kay, K., *Arch. Ind. Hyg. and Occupational Med., 8,* 70 (1953).
72. Kay, K., *Can. J. Public Health, 46,* 273 (1955).
73. Kay, K., Monkman, L., Windish, J. P., Doherty, T., Pare, J., and Racicot, C., *Arch. Ind. Hyg. and Occupational Med., 6,* 252 (1952).
74. Kewitz, H., and Wilson, I. B., *Arch. Biochem. and Biophys., 60,* 261 (1956).
75. Khairy, M., Ph.D. Thesis, University of London, 1956.
76. Klimmer, O. R., *Arch. exptl. Pathol. Pharmacol., 227,* 183 (1955).
77. Klimmer, O. R., and Pfaff, W., *Arzneimittel-Forsch., 5,* 584 (1955).
78. Klimmer, O. R., and Pfaff, W., *Arzneimittel-Forsch., 5,* 626 (1955).
79. Kohn-Abrest, M. E., *Ann. méd. légale criminol., police sci., med. sociale, et toxicol., 33,* 236 (1953).
80. Krone, H. A., and Nilles, H., *Zentr. Arbeitsmed. u. Arbeitschutz., 4,* 71 (1954).
81. Larson, P. S., Hennigar, R., Finnegan, J. K., Blackwell Smith, R., and Haag, H. B., *J. Pharmacol. Exptl. Therap., 115,* 408 (1956).
82. Laug, E. P., Kunze, F. M., and Prickett, C. S., *Arch. Ind. Hyg. and Occupational Med., 3,* 245 (1951).
83. Leach, P. H., *Calif. Med., 78,* 491 (1953).
84. Lehman, A. J., *Assoc. Food & Drug Officials U. S. Quart. Bull., 18,* 87 (1954).
84a. Lewis, S. E., and Fowler, K. S., *Nature, 178,* 919 (1956).
85. Lieben, J., Waldman, R. K., and Krause, L., *Arch. Ind. Hyg. and Occupational Med., 6,* 491 (1952).
86. Lieben, J., Waldman, R. K., and Krause, L., *Arch. Ind. Hyg. and Occupational Med., 7,* 93 (1953).
87. Limperos, G., and Ranta, K. E., *Science, 117,* 453 (1953).
88. Livadas, G. A., *Riv. malariol., 33,* 23 (1954).
89. McGee, L. C., Reed, H. L., and Fleming, J. P., *J. Am. Med. Assoc., 149,* 1124 (1952).
90. McGregor, W. S., Radeleff, R. D., Claborn, H. V., and Bushland, R. C., *Agr. Chemicals, 10,* 34 (1955).
91. March, R. B., Metcalf, R. L., Fukuto, T. R., and Maxon, M. G., *J. Econ. Entomol., 48,* 355 (1955).

92. Mattson, A. M., Spillane, J. T., Baker, C., Pearce, G. W., *Anal. Chem.*, *25*, 1065 (1953).
93. Metcalf, R. L., March, R. B., Fukuto, T. R., and Maxon, M. G., *J. Econ. Entomol.*, *48*, 364 (1955).
94. Michaux, P., Thiodet, J., and Deyme, H., *Arch. maladies profess. méd travail et sécurite socialé*, *15*, 357 (1954).
95. Mosna, E., and Alessandrini, M., *Riv. parassitol.*, *15*, 543 (1954).
96. Mousselon, J., and Tolot, F., *Arch. maladies profess. méd. travail et sécurité sociale*, *14*, 70 (1953).
97. Murtha, E. F., McNamara, B. P., Edberg, L. J., Bergner, A. D., and Willis, J. H., *J. Pharmacol. Exptl. Therap.*, *115*, 291 (1955).
98. O'Brien, R. D., and Spencer, E. Y., *J. Agr. Food Chem.*, *1*, 946 (1953).
99. Pearce, G. W., Mattson, A. M., and Hayes, W. J., *Science*, *116*, 254 (1952).
100. Pest Infestation Laboratory, Annual Report, D.S.I.R., 1955.
101. Petry, H., *Zentr. Arbeitsmed. u. Arbeitschutz.*, *2*, 107 (1952).
102. Pollock, R. W., *Northwest Med.*, *52*, 293 (1953).
103. Pribilla, O., *Arch. Toxikologie*, *15*, 210 (1954).
104. Price, D. E., *Agr. Chemicals*, *9*, 34, 117 (1954).
105. Princi, F., *Modern Sanitation*, *5*, No. 12 (1953).
106. Princi, F., *Agr. Chemicals*, April 1954.
107. Princi, F., *World Crops*, August 1954.
108. Princi, F., and Spurbeck, G. H., *Arch. Ind. Hyg. and Occupational Med.*, *3*, 64 (1951).
109. Roche, L., and Tolot, F., *Arch. maladies profess. méd. travail et sécurité sociale*, *15*, 55 (1954).
110. Sassi, C., *Med. lavoro*, *43*, 210 (1952).
111. Sassi, C., Zurlo, N., Bartalini, E., and Metrico, L., *Med. lavoro*, *46*, 251 (1955).
112. Schaefer, R. A., and Vance, G. H., *Arch. Ind. Health*, *7*, 193 (1953).
113. Schmiedeberg, J., and Wasserburger, H. J., *Anz. Schadlingskunde*, *26*, 129 (1953).
114. Sheehan, H. L., Summers, V. K., and Nichols, J., *Lancet*, *1953*, *I*, 312.
115. Simmons, S. W., and Hayes, W. J., *Soap Sanit. Chemicals*, December 1951.
116. Stoner, H. B., *Nature*, *172*, 1044 (1953).
117. Sumerford, W. T., Hayes, W. J., Johnston, J. M., Walker, K., and Spillane, J., *Arch. Ind. Hyg. and Occupational Med.*, *7*, 383 (1953).
118. Symanski, H., *Arch. Gewerbepathol. Gewerbehygiene*, *13*, 573 (1954–5).
119. Tavernari, A., Bonoli, U., and Cavallini, A., *Med. lavoro*, *46*, 270 (1955).
120. Teleky, L., *Arch. Gewerbepathol. Gewerbehygiene*, *13*, 313 (1954–5).
121. Thomas, G., and Lafontaine, A., *Arch. belges méd. sociale, hyg., méd., travail et méd. legale*, *13*, 410 (1955).
122. Treon, J. F., and Cleveland, F. P., *J. Agr. Food Chem.*, *3*, 402 (1955).
123. Treon, J. F., Cleveland, F. P., and Cappel, J., *Arch. Ind. Health*, *11*, 459 (1955).
124. Troisi, F. N., *Folia Med. (Naples)*, *36*, 881 (1953).
125. Truhaut, R., Vitte, G., Boussemart, E., and Epee, P. L., *Arch. maladies profess. méd. travail et sécurité sociale*, *13*, 561, 567, 570 (1952).

126. Upholt, W. M., Quinby, G. E., and Batchelor, G. S., *Proc. Wash. State Hort. Assoc. 50th Ann. Meeting.*
127. Vandekar, M., and Heath, D. F., *Biochem. J.*, in press.
128. Waldman, R. K., Lieben, J., and Krause, L., *Arch. Ind. Hyg. and Occupational Med.*, *9*, 37 (1954).
129. Walker, K. C., Goette, M. B., and Batchelor, G. S., *J. Agr. Food Chem.*, *2*, 1034 (1954).
130. Weikel, J. H., *J. Pharmacol. Exptl. Therap.*, *116*, 60 (1956).
131. Weinbach, E. C., *J. Biol. Chem.*, *210*, 545 (1954).
132. Wilson, I. B., *J. Biol. Chem.*, *199*, 113 (1952).
133. Wilson, I. B., and Meislich, E. K., *J. Am. Chem. Soc.*, *75*, 4628 (1953).
134. Winteringham, F. P. W., Personal communication. Now published, see ref. 84a.
135. Winteringham, F. P. W., *J. Sci. Food Agr.*, *6*, 269 (1955).
136. Winteringham, F. P. W., and Barnes, J. M., *Physiol. Revs.*, *35*, 701 (1955).
137. Wolfsie, J. H., and Winter, G. D., *Arch. Ind. Hyg. and Occupational Med.*, *6*, 43 (1952).
138. World Health Organization. Statement by Director General 17th Session Executive Board, Geneva, 1956.
139. Zurlo, N., Sassi, C., and Metrico, L., *Med. lavoro*, *45*, 533 (1954).

# THE CHEMISTRY AND MODE OF ACTION OF HERBICIDES

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## I. Introduction

Announcement of 2,4-D and 2,4,5-T as translocated herbicides in 1944 (33) ushered in the modern era in the use of herbicides. To be sure the dinitro compounds had been known since the middle 30's

(56), and arsenic, chlorates, and boron compounds for even longer. Soil sterilization by the latter three compounds had become common practice in some western areas (46), and Sinox (sodium dinitro-*o*-cresylate) was gaining in acceptance being used in peas, flax, onions, garlic, and grass seed crops as well as in the cereals, barley, oats, and wheat.

The new auxin-like chemical 2,4-D, however, incorporated properties hitherto unheard of in weed control chemicals. Not only did it have contact action on the sprayed foliage; it translocated from the tops into the roots of perennial weeds; it was selective against many broad-leafed weeds in cereal and grass crops; it was absorbed from the soil and hence could be used by the pre-emergence method; and it was a cheap and potent chemical capable of controlling weeds at costs of around $1.00 per acre for the toxicant. Furthermore, being translocated, it soon proved useful in low volume application at a volume rate of one gallon or less per acre; it was ideal for airplane application, a method popularized by DDT usage during the World War II. And it was highly advertised as the "hormone" weed killer; it caught the public fancy and became a vogue among farmers and gardeners.

The versatility of this compound quickly became apparent. Suggested as a translocated herbicide in this country and as a selective soil sterilant in Britain (49), 2,4-D was soon found to be an ideal selective herbicide. Its use as a pre-emergence material followed (3–5). Since then it has been used in higher dosage as a soil sterilant (6).

While these developments were taking place considerable research and a tremendous amount of field testing has been done on the formulation of 2,4-D. First used in a Carbowax mixture it was rapidly tried as the sodium salt, as alkyl esters, as ammonium and amine salts and in a great variety of low volatile esters. And added to this array were the 2-methyl-4 chloro (MCP) analogue, the 2,4,5-T form, and more recently propionic, butyric, caproic, and octanoic acid compounds (55).

While all this development has been occurring older herbicides have appeared in new formulations and many new and interesting compounds have been introduced.

First should be mentioned the pentaborate compounds, the high solubility of which enabled formulators to make a borate-chlorate combination safe from the fire hazard standpoint and soluble enough to be applied as a spray at relatively low volume rates. Later introduction of the metaborates has furthered this trend.

The dinitro compounds have been formulated as oil solutions of the native substituted phenols. These are general contact materials useful as fortifiers for oils and oil-water emulsions. By substituting a secondary butyl chain in place of the methyl group of the cresol the toxicity of dinitro compounds has been materially increased (10). And the amine salts of dinitro secondary butyl phenol have been introduced as pre-emergence materials for application to the soil. Pentachlorophenol and its salts are used in similar ways.

As might be expected this tremendous upsurge in the application of herbicides has stimulated interest in possible new compounds, and screening and testing of herbicidal chemicals has proceeded at an accelerated rate. Ammonium sulfamate had been introduced in the late 30's. Carbamates were announced in 1945; isopropyl phenyl carbamate (IPC); later chloro IPC. Then in quick succession came trichloroacetic acid, phenyl mercuric acetate, potassium cyanate, phenoxyethylsulfate and similar compounds, dichloral urea, 3,6-endoxohexahydrophthalic acid and its salts, maleic hydrazide, sodium isopropylxanthate, *N*-1-naphthyl phthalamic acid and its salts, the substituted urea herbicides, sodium 2,2-dichloropropionate (Dalapon), 2,3,6-trichlorobenzoic acid, aminotriazole, the chloro acetamides of Monsanto, the triazines, dithiocarbamates, and others. With so many tested chemicals available and many others on the way, it is apparent that some system of classification is needed to make order out of the confusion.

## II. Classification of Herbicides

The following scheme has proved very useful in classifying herbicides into coherent, related groups.

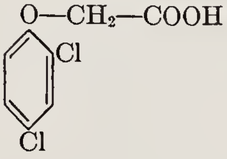
| Selective Herbicides | Nonselective Herbicides |
|---|---|
| 1. Spray applications | 1. Spray applications |
| a. Contact | a. Contact |
| b. Translocated | b. Translocated |
| 2. Root applications | 2. Root applications |

A few methods do not fit this scheme, for instance, the jar method whereby a translocated material is applied by soaking the tops in a solution in a can or jar. Another is the cut surface application of

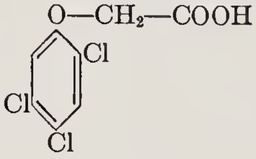
2,4-D or 2,4,5-T whereby the undiluted amine salt formulation is squirted into cuts made through the bark of a tree near the base. A third is the aromatic solvent application, made to aquatic vegetation through the ambient water. Practically all other methods fit the above scheme and when they are used with this scheme in mind the applicator is aiming his treatment at a definite mechanism.

## III. The Phenoxy Herbicides

The principal members of this family are 2,4-D (I), 2,4,5-T (II), and MCP (III). Two others of increasing interest are 4-chlorophenoxyacetic acid (IV), and 3,4-D (V). Each of these compounds can be applied as the suspended acid, that is as a finely ground form of the original acid formulated as a wettable powder or as a paste that can be thinned to any convenient strength. The acids are low in volatility and solubility so that application to the soil as soil sterilants or pre-emergence herbicides in regions of heavy rainfall may well be made in the suspended form.



(I)



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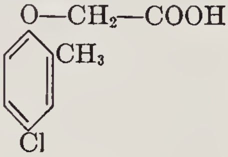

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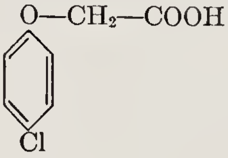

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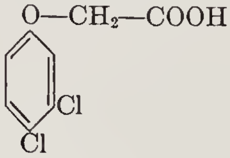


(V)

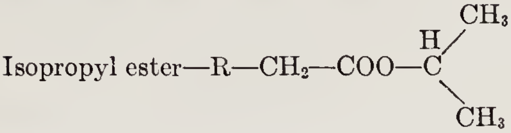
Each may also be formulated as the sodium or potassium salt, the ammonium salt or an amine salt, as a short chain alkyl ester, or finally as a long chain alkyl or alkyl ether chain ester. Designating the phenoxy group as R, a few of these are:

*Salts*

Sodium salt—$R—CH_2COONa$
Potassium salt—$R—CH_2COOK$
Ammonium salt—$R—CH_2COONH_4$
Triethylamine salt—$R—CH_2—COONH(C_2H_5)_3$
Triethanolamine salt—$R—CH_2—COONH(C_2H_4OH)_3$

*Esters*

Methyl ester—$R—CH_2—COOCH_3$



Isopropyl ester—$R—CH_2—COO—C(H)(CH_3)_2$

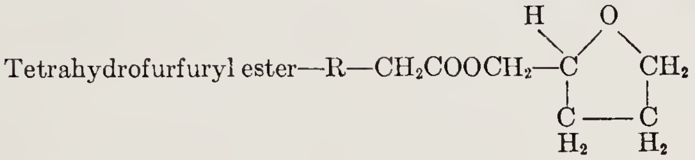
Butyl ester—$R—CH_2—COO—C_4H_9$

Octyl ester—$R—CH_2—COO—C_8H_{17}$

*Heavy or Low Volatile Esters*

Butoxyethanol ester—$R—CH_2COOC_2H_4—O—C_4H_9$

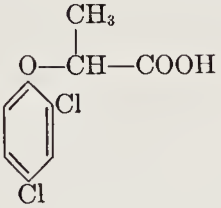
Propylene glycol butyl ester—$R—CH_2—COOCH_2CH(OH)CH_2OC_4H_9$



Tetrahydrofurfuryl ester

It should be realized that the above represent only a few of the commoner compounds that have been used in tests or in the field. Almost innumerable alkyl and alkanol amine salts may be synthesized; the same is true of the esters. It should also be realized that R in the above formulas could be 2,4-D, MCP, 2,4,5-T, 3,4-D, or the parachlorophenoxy acetic acid radical.

Next it should be pointed out that in the place of the phenoxy acetic acids, the phenoxy propionic acids can be substituted representing a series of compounds potent against certain woody plants, including a number of oak species. The basic compound is 2,4-dichlorophenoxypropionic acid (VI). Formulation of this family



(VI)

offers all of the possibilities illustrated for the acetic acid derivatives above.

Finally the very interesting series pointed out by Synerholm and Zimmerman (52) as offering possibilities for beta oxidation to acetic acid should be described. The above workers tested a long series of 2,4-dichlorophenoxy alkyl acids running from the acetic acid to the octanoic acid. They made the important observation that with tomato epinasty those having an even number of carbon atoms in the acid chain were active whereas those having an odd number were not. To explain this they suggested that the test plant must possess a beta oxidation enzyme system capable of degrading the various chains down to the 2-carbon acetic acid. The odd-numbered chains would be degraded to phenol which is impotent as a growth regulator.

In testing a great number of phenoxy compounds Wain (55) found that wheat sections would respond to the higher even-numbered members of this series whereas pea stem sections would not. However, if he placed wheat sections in the solutions and later added pea stem sections, these would respond. From this he reasoned that some plants possess a beta oxidation mechanism capable of degrading the even-numbered members to acetic acid, while other plants did not. Consequently if weeds could be found having the beta oxidation mechanism and crops that lacked it, a new and effective mechanism of selectivity would be available.

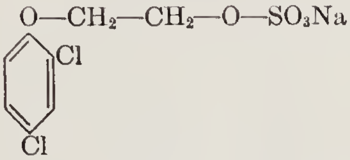
Testing proved this surmise to be true and now a number of the butyric, caproic, and octanoic acids are being extensively tested. A few sensitive weeds are nettle, Canada thistle, fumitory, knotweed, pigweed, lamb's quarters, and wild mustard. A few tolerant crops are celery, red and white clover, alfalfa, carrot, parsnip, and flax.

Finally it should be mentioned that all the above ring substitutions may be used in the three acid forms just mentioned and all these in turn may be formulated as acids, salts, and esters. Wain has tested many of these compounds. Gamma phenoxy butyric acid is $R—CH_2—CH_2—CH_2—COOH$.

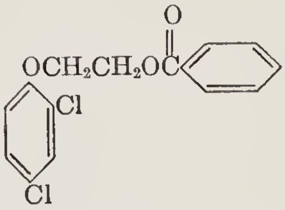
With respect to the scheme of classification given earlier, all the phenoxy compounds are selective herbicides and they may be applied as translocated sprays which means that they can be used in low volume applications. They are also selective as root applications and hence can be used pre-emergence. In general they are selective against broad-leafed weeds in cereal or grass crops (including corn, milo, and sugar cane). They are most toxic to very young tender plants. And a few broad-leafed crop plants including clover, flax, strawberries, and coffee tolerate low dosages.

In general the 2,4,5-T forms are more toxic to woody or bushy species than to herbaceous plants. MCP is more selective than 2,4-D and is particularly useful in cereal crops under seeded to legumes. It is also proving superior to 2,4-D for weed control in rice for this crop is often injured if treated with 2,4-D during extremely hot weather (35a).

An additional group of compounds related to those given above are the esters of chlorophenoxy alcohols, 2,4-dichlorophenoxy ethyl sulfate, sodium salt (VII), Crag 1 or SES; 2,4-dichlorophenoxy ethyl benzoate (VIII), Sesin; and 2,4,5-trichlorophenoxy ethyl sulfate, sodium salt (IX), Natrin. These compounds have little or no contact action. When incorporated in the soil they are broken down and converted to the acetic acids and hence become effective toxicants.



(VII)



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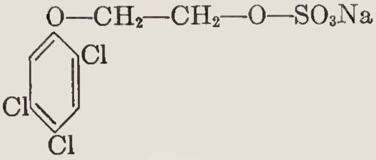

(IX)

### A. MECHANISM OF ACTION OF PHENOXY COMPOUNDS

The complete action of the hormone-like herbicides consists of (1) absorption, (2) translocation, and (3) action at the cellular or subcellular level at some critical sites within the plant.

#### 1. *Absorption*

Absorption involves penetration of the cuticle and migration across epidermis and mesophyll to the phloem. With the dinitro and the phenoxy compounds it has been shown that pH of the spray formulation has a definite effect upon penetration of the cuticle. Buffering on the acid side increases penetration and the response of bean plants by the bean bending test is shown in Table I (14). These data show that the amount of 2,4-D[1] entering the leaf from a given application dosage increases through the range pH 10 to pH 2. When the formulation is more acid than pH 2 there is rapid injury in the treated area and bending is distinctly less.

---

[1] The term 2,4-D will be used throughout this discussion in the generic sense, that is, as typical of the chlorophenoxy compounds.

TABLE I

EFFECT OF pH OF SPRAY ON PENETRATION

| pH of soln. | Hours after application | | | | | | |
|---|---|---|---|---|---|---|---|
| | 3 | 4 | 5 | 6 | 7 | 8 | 28 |
| 0.5 | 1.0 | 3.0 | 1.5 | 2.5 | 7.0 | 10.0 | 11.0 |
| 1.0 | 2.0 | 2.5 | 1.5 | 1.5 | 4.5 | 5.5 | 7.0 |
| 2.0 | 5.5 | 21.5 | 65.5 | 100.5 | 102.5 | 94.0 | 91.5 |
| 3.0 | 4.5 | 27.0 | 58.0 | 88.5 | 94.0 | 87.5 | 92.5 |
| 4.0 | 0.0 | 15.0 | 54.5 | 94.5 | 96.0 | 81.0 | 84.0 |
| 5.0 | 0.0 | 11.0 | 46.0 | 87.5 | 100.0 | 88.0 | 86.0 |
| 6.0 | 1.5 | 12.0 | 44.5 | 90.5 | 100.0 | 91.0 | 79.5 |
| 7.0 | 0.0 | 1.0 | 9.0 | 32.0 | 70.5 | 56.5 | 82.5 |
| 8.0 | 0.0 | 1.0 | 1.0 | 10.0 | 24.5 | 72.5 | 76.5 |
| 9.0 | 0.0 | 0.0 | 1.5 | 10.5 | 39.0 | 70.0 | 91.5 |
| 10.0 | 0.0 | 0.0 | 1.0 | 12.5 | 40.0 | 68.0 | 86.5 |

This response, first noticed with dinitro compounds (18), was interpreted as indicating that the undissociated parent acid dissolves in, and moves through the fatty cuticle more readily than does the ion with its negative charge. The same generalization can be applied to the phenoxy compounds (11). Continued testing indicates that it holds for these two groups of compounds, and for others that are acidic in nature (i.e., Dalapon). Applied in formulation practice it has resulted in several suspended acid and emulsifiable acid products that have distinct advantages against hard-to-kill species.

In considering pH relations of spray materials it is interesting to note that a 2,4-D formulation containing the free acid has a pH of around 3.3 when applied to the cuticle. Upon entering and migrating to the epidermis the 2,4-D molecule finds itself in a medium around pH 5.5; a certain degree of dissociation must take place. As it progresses across the mesophyll to the phloem the pH again changes to a value of around 7.0. Dissociation here must be almost complete. This dissociation process undoubtedly steepens the gradient for movement from cuticle to phloem. It has no detrimental effect because it is only penetration of the cuticle that is benefited by the low degree of dissociation; once this fatty layer is traversed water solubility and hence ionization enhance movement. This characteristic of the 2,4-D molecule is probably one reason for its unique quality as a translocated herbicide.

### *2. Translocation*

Once the 2,4-D ion reaches the phloem it seems well established that it moves with food materials within that tissue. (For a review of the evidence for this, see reference 13,15–17.) Movement is from regions of food synthesis to those of food utilization; it occurs only if photosynthesis is going on or if carbohydrate reserves are being hydrolyzed and moved; it can be induced in starved plants by any one of a number of sugars applied to the leaf; work with $C^{14}$ labeled 2,4-D shows that rapid transport is limited to the sieve tubes.

Tracer studies show that 2,4-D*[2] is absorbed and moved most rapidly by young plants; absorption and translocation are not correlated with dosage applied; small amounts of the tracer are metabolized by the indicator plants. Water deficit eliminated the bean bend response but did not affect translocation of the 2,4-D*; it did reduce the final response by roots to the herbicide.

### *3. Absorption by Roots*

2,4-D applied through the culture solution or through the soil is readily absorbed, presumably in the ionic form. It is rapidly moved in the transpiration stream to the leaves; movement to buds may follow after an appreciable time lag, presumably after movement into and along the phloem. Where 2,4-D is being used as a pre-emergence treatment, the extent of leaching can be somewhat determined by the form applied. In regions of heavy rainfall and in sandy soils the parent acid, being of low solubility, is more persistent than the salts; in dryer regions the salt form is preferred.

### *4. Final Action*

There is a tremendous literature on the mechanism of action of growth regulators and 2,4-D is one of the prominent compounds considered (15,38,54). Though this is not the place to consider such mechanisms in detail, one of the more attractive schemes, that of 2-point attachment (39), has probably received the most attention (38).

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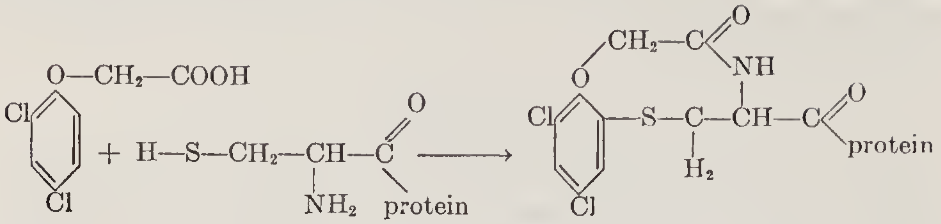
[2] This symbol is used to designate $C^{14}$-labeled 2,4-D.

This mechanism postulates a reaction such as:



In this a 2-point reaction takes place between the regulator and a cysteinyl unit of a protein.

According to recent publications on growth regulator activity (43,44), the basic response on the part of plant tissue is a relaxation of the cell walls, reducing wall pressure and allowing osmotic uptake of water with a resultant increase in volume of the cell. If this is true then it might be expected that the above regulator-protein complex might constitute an enzyme or a part of an enzyme system instrumental in cell wall growth. In this connection the finding of Neeley *et al.* (41) that plants treated with 2,4-D had an increase in pectin methoxylase is of interest.

Although theories of growth regulator action involving stimulation or inhibition of growth are pertinent to the problem of herbicidal activity they do not provide a satisfactory explanation of toxicity and lethal effects. More important here would seem to be evidence that 2,4-D may be metabolized in plants accompanied by an increase in energy-rich phosphate bonds. If these are dissipated in a wasteful oxidation of carbohydrate reserves without the synthetic processes normally included in the respiration cycle, then such a mechanism might explain at least a part of the action of 2,4-D on plants.

Before closing this discussion it might be well to point out that the action of the hormone-like weed killers essential to their function is the destruction of meristems that normally account for growth and development of the plant. Mature leaves, stems, and roots may be killed, but without destruction of buds; perennial weeds recover. For this reason it seems reasonable that any testing method useful in the assay of herbicidal toxicity of such compounds should evaluate meristem inhibition and death. For this reason the bean shoot inhibition technic and various procedures involving total destruction of tissues are of greater value than Avena curvature, coleoptile length growth, pea stem curvature, or bean bending that simply evaluate the effects of the substance on length growth of parenchyma cells.

Again, in thinking of mechanism, it might be well to consider the great array of plants that respond to 2,4-D, the wide range of response from simple bending to structural deformation to death, the highly specific nature of some responses and the almost nonspecific nature of others, and the number of chemicals that cause at least some of the responses. From this consideration it seems obvious that the affected responses must be very basic in nature. As indicated earlier (12,15) it seems that the action of 2,4-D must affect the plant at a very early stage in the respiration process, that formative effects take place very soon after young cells leave the meristems, and that the processes disturbed are essential to the very life of the plant. Certainly the primary bending responses can have little to do with these final lethal effects; it seems much more likely that essential, enzyme-controlled steps in the initial stages of respiration must be involved. One such process is the concurrent oxidation of acetaldehyde to acetic acid and reduction to alcohol. Freed and Remmert (26) have shown that the enzyme system involved in these reactions is inhibited *in vitro* by 2,4-D.

### *5. Formulation*

With a group of compounds such as the chlorophenoxy family knowledge of the chemistry of the various members coupled with data on plant specificities and responses allows the formulator to do many things that result in benefits to the user. Considering first the salts, the sodium salt of 2,4-D, used very extensively the first year, has now almost passed out of the picture. However, because of specificities it is still employed in controlling wild morning glory in asparagus in the mature fern stage because it controls the weed with minimum damage to the crop. Likewise it is used to control one species of Ribes in the Sierra Nevada Mountains of California because it is more effective than the amine salts and it must be applied in water solution.

The isopropyl ester of 2,4-D is the form sold in greatest quantity because it is most effective against broad-leafed weeds in wheat and other small grains. Most of it is applied at low volume rates with diesel oil as the carrier. Against these weeds there is no requirement for extensive translocation into the roots.

Where volatile esters are prohibited, i.e., in California, the amine salts are most favored for weed control in grain crops. They are convenient; water as a diluent is cheap; volume can be kept within a practical range for airplane application (5–10 gal./acre); and results on mustards and aquatic weeds of rice are satisfactory. Sequestering agents are included where hard waters prevail.

Ester forms of 2,4-D early proved their superiority as far as absorption is concerned; dosage could be reduced to about half that of salts; application could continue up to and even during rainfall. Translocation of the light alkyl esters however was not effective; they are too aliphatic (oil-like) to part into the highly aqueous cell sap of mesophyll and phloem.

With prohibition of use of the light esters manufacturers soon supplied heavy esters of low volatility. These materials soon solved most of the problems of injury caused by vapor drift. They retained the superior wetting and penetration properties of the esters on hard-to-kill weeds such as *Amsinckia* species, *Salsola kali,* and *Centaurea solstitialis.* They did more than this. They brought back the superior translocating properties displayed by the original acid-in-Carbowax formulation.

As one examines the alcohol chains used in most of the heavy esters of 2,4-D and 2,4,5-T he can see the reason for this. Most of them are comprised of short alkyl chains connected by ether linkages. The former lend oil solubility to these esters, the latter water solubility. And the balanced solubility provides for ready, ordered penetration of the cuticle and then enough water solubility to allow the molecules to part into the aqueous phase and move on into the phloem. Contact injury is not severe.

The butoxy ethanol ester (p. 43) illustrates one of the simplest and most effective of these. A good many similar ones are on the market. An exception seems to be the tetrahydrofurfuryl ester (p. 43). This ester is composed of two ring structures and it resembles, at least superficially, the sucrose molecule. For this reason it seemed, at first thought, to be a promising compound. Many tests, however, have found it wanting and a little chemical reasoning provides the answer. Apparently for rapid absorption an herbicide should have small exposed groups of either high water or oil solubility or both. Sucrose is not readily absorbed through the cuticle and several 2,4-D diesters of the glycols have proved disappointing. Apparently the tetrahydrofurfuryl ester is not readily taken into the plant and so it is not effective.

Another type of compound that may prove disappointing is the heavy ester with a long alkyl chain. Though heavy enough in molecular weight to be relatively nonvolatile such esters are highly fat soluble and they probably will not have sufficient polarity to part from the lipoid phases of the tissues. Several such esters are on the market, but they have not been sufficiently tested to provide a satisfactory evaluation.

From this discussion it should be apparent that the formulator is making a compromise between fat and water solubility attempting to provide adequate but ordered penetration of the surface layers followed by proper partition to and migration in a highly aqueous medium. For success the user is demanding that a living tissue system in the plant (phloem) transport a toxic material without stoppage, at least in the early stages. Too rapid penetration means high concentration in the phloem resulting in injury; too slow penetration allows time for drying of the chemical on the foliage and metabolism of the toxicant within living cells resulting in detoxification. High lipoid solubility makes for too rapid penetration, also for poor partition to the mesophyll and phloem. High water solubility usually implies ionization and the negatively charged phenoxyacetate ion is repelled by the cuticle in the presence of water; in the absence of water the salts tend to crystallize out on the surface and absorption ceases.

In addition to the toxicant most formulations include surfactants, cosolvents, and additives of various sorts. It is most important that none of these be highly toxic such, for instance, as highly aromatic oils.

Gast and Early (27) have a recent publication presenting data on phytotoxicity of 77 emulsifiers and 86 solvents used in insecticide formulation. Six plants: sweet corn, cucumber, cotton, lima bean, tobacco, and tomato were used in the tests. The emulsifiers were used at 1.0 and 0.1% concentrations in water by a leaf dip technic and toxicity averaged for three replicates was assayed on an arbitrary scale of 0 for no injury to 4 for very severe injury or death. Tests were read after 7 days. Since these same emulsifiers might be used in herbicide formulations it is interesting to note the wide variation in toxicity that they exhibit. For example of 10 materials carrying the Atlox designation toxicity indices averaged over the 6 test plants varied from 2.25 to 0.22 at 1.0% concentration; from 0.94 to 0 at 0.1% concentration. Sixteen Emcol products varied from 1.87 to 0.32 at 1.0%; from 0.33 to 0 at 0.1%. Five Igepals varied from 3.13 to 1.42 at 1.0%; from 0.66 to 0 at 0.1%. Eleven Tritons varied from 3.18 to 0.11 at 1.0%; from 1.25 to 0 at 0.1%. Toxicity to roots varied from 4.0 to 0 at 1.0%; a few were rated at 4.0 at 0.1%.

Tomato roots were more sensitive than tobacco. Creaming time for emulsions of three oils with which the 77 emulsifiers were combined ran from 1 minute to more than 60 minutes. The authors state that in general the more effective emulsifying agents proved to be the more phytotoxic. They relate this to their better ability to penetrate the leaf tissues and this seems reasonable because high stability of an emulsion means high concentration at the interface and this property would bring about high concentration of the emulsifier at the cuticle: water interface, hence a high gradient tending to move the emulsifier into the leaf. Solubility in the cuticle should parallel solubility in the oil being emulsified which in turn is a primary requisite for effective stabilization.

Concerning the solvents, these were emulsified, each with the use of the two emulsifiers Triton X-177 and Atlox 3335. These were two of the emulsifiers that showed low toxicity in the emulsifier tests. The solvents were emulsified and the plants treated by the leaf dip method. The solvents were used at 5.0% and 0.5%. Solvents showing no toxicity at the 5.0% concentration are as follows: normal and isobutyl alcohol, cyclohexanone, diacetone alcohol, dichloroethyl ether, dioxane, ethyl acetate, ethyl alcohol, ethylene dichloride, methyl alcohol, methyl ethyl ketone, isopropylacetate, normal, and isopropyl alcohols. A few producing high toxicity are as follows: tributylphosphate, 4.0; dibutoxy tetraglycol, 3.91; 2 ethyl hexane diol 1,3, 3.64; Sohio insect oil 30, 3.27; Amsco ink oil 10-550, 3.27; Velsicol AR-60, 3.06; benzyl alcohol, 3.0. In general the petroleum distillates are more toxic than the other types of solvents. The acetates, alcohols, ketones, and ethers were in general nontoxic if there were less than 4 carbons in the carbon chain. It is very interesting that tributyl phosphate was the most toxic solvent tested because at one time it was highly recommended as a cosolvent in the formulation of 2,4-D esters.

Some of the newer formulations carry a high content of surfactant and recent research and field tests indicate that this may increase penetrability of the cuticle as well as lower interfacial and surface tension. In this connection it should be obvious that any action of a component of a formulation to increase penetration of the cuticle should be compatible with the toxicant to the extent that it is the toxicant and not some other compound to which the cuticle is rendered permeable. This is largely a matter of solubility and partition.

Filming agents (i.e., oils or glycols), sequestering agents, and cosolvents enter into many modern formulations. It should be obvious that all such additives should be field tested for toxicity, compatibility, and necessity before they are put into a product. There is evidence that a good many formulations have been concocted largely for economy and convenience. These are necessary but not always essential features of a formulation.

### *6. Application Methods*

With knowledge of the physiology of the action of a herbicide it is possible to arrange the application to meet specific requirements of the material being used. As examples pentachlorophenol and the dinitro compounds require minimum temperature of around 60°F. for satisfactory action. The phenoxy compounds will kill cool weather plants at temperatures around 40–50°F.; against warm weather summer annuals they are more effective around 70°F. Most of the substituted phenol herbicides are most effective during moist weather and medium to warm temperatures. Against annual weeds these requirements should be observed.

For satisfactory translocation into roots of perennials 2,4-D should be applied after leaves have attained full size but before they become hardened by hot dry weather. Food movement is active from this time on through the summer period but spray application should be made during early bloom on unirrigated fields because depletion of soil moisture results in termination of root growth, and response by the roots fails. On evergreen perennial brushy species in California foothills, sprays applied in February and March are most effective because the leaves are healthy, foods are moving, and roots are growing in the moist soil. Later, movement of foods may be largely to new twigs, leaves, and fruits; roots in moisture-depleted soil stop growing and do not respond.

### *7. Soil Treatment*

As mentioned earlier, 2,4-D is readily absorbed from the soil. It is used in two ways: at low dosage as a pre-emergence application; at high dosage as a temporary soil sterilant against perennial weeds. In the latter method mixtures of 2,4-D with borax or other boron compounds is proving very effective. It is postulated that the boron compounds have conserving action on the 2,4-D resulting from their biostatic activity on soil organisms (50).

## IV. Oils

Diesel oil and low grade distillates have long been used as general contact sprays against weeds. In the early 40's stove oil was found to be selective against weeds in carrot crops. Research soon proved that lighter fractions of the Stoddard Solvent type are superior; they are more selective and they are more volatile; less oil flavor is left on the crop. By 1947 a number of oil companies were selling carrot oils; in the East, Sovasol No. 5, Varsol No. 2, mineral spirits, and Stoddard Solvent have been used; in the West, Shell No. 10, Seaside Selective Weedkiller, Pentox No. 1, Avon Arland No. 76, Beacon Selective No. 5, and others are the products on the market. These oils have A.P.I. gravity ratings of 40–44°, viscosities below 30 seconds, flash points above 100°F., boiling ranges between 300 and 400°F., and aromatic contents in the range of 14 to 25%.

Meanwhile testing proved that certain heavy aromatic fractions were far superior to diesel oil as general contact herbicides and special oils were produced and sold as weed killing oils. In general these oils have gravity ratings in the 25–35° range, viscosities around 50 seconds, flash points around 200°F. or above, boiling ranges between 500° and 700°F., and aromatic contents from 60 to 90%. They are comprised of low grade distillates, often high in sulfur or nitrogen, or of the bottoms from the catalytic cracking of crude oils in gasoline production. Many of them are fairly high in di- and tricyclic aromatics, compounds that, because of high toxicity, low volatility, and great creeping power, are able to penetrate deep into the roots of perennial grasses and bring about a type of systemic killing. A few such oils are Richfield A, Shell 20, Pentox 2, Annalos 11, Controlol No. 4, and Hy Kil Weed Killer No. 6.

Another general contact oil of lower viscosity and higher volatility is an Edleanu extract, produced by solvent extraction of kerosene distillate. One such product Annalos 7 is largely used for preharvest desiccation of clover, alfalfa, and milo.

Aromatic solvents combined with emulsion stabilizers are widely used to control aquatic weeds in irrigation ditches. These are paint thinners of the xylene type and they should conform approximately to the following specifications: Flash point not less than 80°F.; ASTM D-86, distillation °F., starting point 278; 10%—286; 90%—395; end point not above 420. ASTM D-875, aromatics not less than 85%. Such oils may be purchased with the stabilizers included and they are used by pumping them under pressure through nozzles below the water surface. They form a milky emulsion and they are absorbed from the water by the weeds causing rapid destruction of the latter.

One additional use of oils in weed control is in the form of solvents or diluents in formulations of 2,4-D and other organic herbicides. For use in pre-emergence materials and formulations of defoliants and general contact sprays toxic oils may be used. In translocated herbicides to be used on perennial weeds, brush and trees, such oils should be nontoxic.

### A. MECHANISM

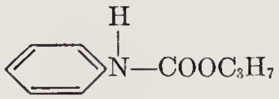
Oil toxicity results from the denaturation of the plasma membranes of the cells resulting from solubilization of the oil (45). This response follows treatment by liquid oil or by oil vapor and the first result is a greatly increased permeability followed by leakage of cell sap into intercellular spaces, death of cells, and drying of the tissues (20).

Work by Currier and Peoples (21) indicates that oil toxicity is closely related to oil-air distribution values when plants are treated with hydrocarbon vapors and when vegetable oils are used in calculating the distribution coefficients.

Calculation of concentration values in cell lipid from distribution coefficients gives rather constant minimum lethal values of about 2 mols. per liter for barley and 3 for carrot; differences between oils must be ascribed to physical factors involved in movement of molecules to and retention at sites of action. Toxicity seems to result from accumulation of the oil in the cell lipids resulting in a cytolytic action; the ectoplast is encountered first and its destruction results in the rapid increase in permeability and sap leakage observed.

## V. Carbamates

### A. IPC



(X)

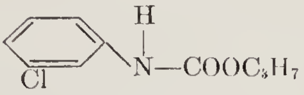
Templeman and Sexton (53) found isopropyl *N*-phenyl-carbamate (X) active as a grass killer and suggested its use as a herbicide. They found that concentrations that killed oats and other cereals were harmless to sugar beets, flax, marigolds, rape, and mustard. Allard *et al.* (2), Ennis (23), and Mitchell and Marth (37) found IPC toxic to *Agropyron*, *Oryza*, *Festuca*, *Lolium*, *Zea*, and *Phleum* species, less effective on *Milium*, *Setaria*, and *Sorghum* species, while soybeans and sunflower were resistant. Carlson (7) applied IPC to quack grass and found it ineffective as a spray but active through the soil at concentrations of 500 p.p.m. and above.

Ivens and Blackman (35) showed the action of ethyl phenylcarbamate to be similar to that of colchicine. It caused a disruption of mitosis in treated pea and barley roots with an increase in arrested metaphase figures. In barley roots there was increase in cell size, many polyploid nuclei, varying degrees of chromosome contraction, multinucleate cells, and a great increase in metaphase nuclei compared with anaphase and telophase. Dosages that inhibited length growth interfered with cell division and produced nuclear abnormalities. They postulate that these compounds associate with lipophilic side chains of the proteins in the spindle leading to an intramolecular precipitation, folding of protein chains, and disintegration of the spindle. These observations indicate why foliage applications fail while application to roots through the soil are effective.

Freed (25) recommends in use of IPC on quack grass that the chemical be applied to mature foliage in an oil carrier and that treatment be followed by tillage. Such treatment probably combines the contact effect of the oil with exposure of roots to the IPC following the tillage. Freed found a mixture of maleic hydrazide plus IPC superior to IPC alone. In control of annual grasses Freed recommended treatment in the young seedling stages. Moisture is essential in bringing the chemical in intimate contact with the roots. Tillage also helps where it can be used. IPC is subject to rapid breakdown by microorganisms in warm, moist soil, hence it is most useful in winter and early spring. It is most useful in cool season crops such as spinach, peas, flax, sugar beets, strawberries and cane fruits, onions and bulb crops, and asparagus nurseries.

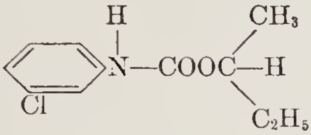
The compound isopropyl *N*-meta-chlorophenyl carbamate (XI) is more resistant to microbial decomposition and it has proved useful in cotton, alfalfa, ladino clover, and asparagus.

### B. CHLORO IPC

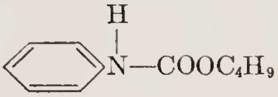


(XI)

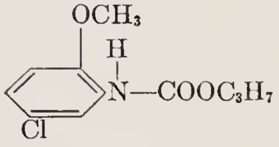
In preliminary trials on some 45 carbamates Shaw and Swanson (47) found sec butyl *N*-(3-chlorophenyl) carbamate (XII) to be most toxic; chloro IPC was second; sec butyl *N*-phenyl carbamate (XIII) was third; isopropyl *N*-(3-chloro-6-methoxyphenyl) carbamate (XIV) was fourth; isopropyl *N*-(3-methylphenyl) carbamate (XV) was fifth; isopropyl *N*-(3,6-dichlorophenyl) carbamate (XVI) was sixth. IPC was eighteenth on their list in terms of effectiveness, chloro IPC second. Numbers four and five of the above group having methoxy and methyl substitutions on the phenyl ring produce chlorosis of the foliage as well as being toxic to roots.



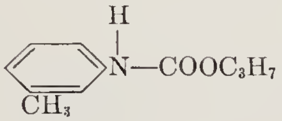
(XII)



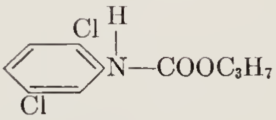
(XIII)



(XIV)



(XV)

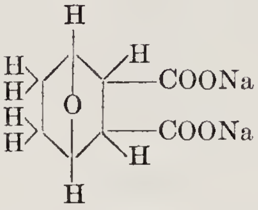


(XVI)

The most recent development of the carbamates has been their pelleting (42) so that they can be applied dry, either by hand, ground applicator, or airplane. The last method being independent of soil conditions will undoubtedly result in great expansion of the use of carbamates, particularly on forage and vegetable crops, because winter or early spring application is often impossible from the ground. IPC, chloro IPC, and mixtures of the two are available in pelleted form. The mixture is recommended as being more general in its effects on grasses, combining the selectivities of the two active ingredients. It is suggested for use against chickweed, ryegrass, pigeon grass, and wild oats in established alfalfa and ladino clover at 5 lb. active ingredient per acre. Against velvetgrass, hairy crabgrass, watergrass, dock, and mustard, 7 to 8 lb. should be used. The mixture is also recommended for control of chickweed, shepherd's purse, and annual grasses in woody nursery plantings, in established cane fruits, and a number of other crops.

IPC and chloro IPC are available as wettable powders and as emulsifiable concentrates. The use of carbamates will undoubtedly increase as more people become familiar with their special properties.

## VI. Endothal

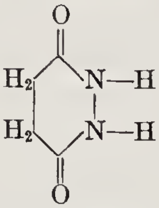


(XVII)

The disodium salt of 3,6-endoxohexahydrophthalic acid (XVII) is a water soluble toxicant used as a defoliant and weed killer. As a defoliant it has a true hormone effect in inducting abscission of leaves at a distance from the point of application.

Endothal is readily absorbed from the soil by roots and carried to the foliage in the transpiration stream, hence it is useful as a preemergence herbicide. Applied as a spray it can be activated with ammonium sulfate and it is selective by differential wetting if used without a surfactant; with a surfactant it is more general in its contact effects. However it has certain selectivities that have to be recognized. It is not translocated downward through the phloem and hence it will not kill perennial weeds by spray treatment. Lamb's-quarters and milkweed are annuals that it does not kill. Knotweed, chickweed, shepherd's purse, and many annual grasses are susceptible. A number of crops are somewhat resistant to herbicidal doses.

## VII. Maleic Hydrazide (MH)



(XVIII)

Maleic hydrazide (XVIII), introduced in 1948, is one of the most interesting compounds in the herbicide field. It is not only a weed killer; it also inhibits growth without causing serious injury and it may find its greatest use as an inhibitor rather than as a herbicide.

MH has been shown to inhibit growth of grass species and many woody plants. It inhibits sprouting of potato tubers, onion bulbs, carrot and sugar beet roots, and topped tobacco shoots. It prevents bolting of radishes, sugar cane, sugar beets, and celery. It slows growth of trees in nurseries, stops growth of cotton shoots, and in sweet potato roots it stimulates shooting at low dosages and inhibits shooting at high. It induces dormancy in strawberry runners and slows maturity of apple fruits. MH has been shown to make wild oats sterile and by proper timing can be used to do this with no harm to barley and some wheat varieties. It prevents seeding of sandbur (*Cenchrus* sp.) and can be used to produce male sterile cucurbit and sorghum varieties. This may prove useful in the production of hybrid seed.

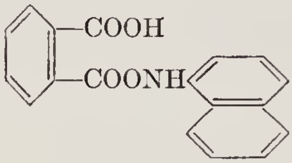
As a weed killer MH is most active against grasses. If quack grass, Bermuda grass, or Johnson grass is sprayed in late spring and then plowed or thoroughly disked, the land may be seeded to a crop with no reduction in yield. It is also effective against wild onions and wild garlic treated in the same way. In these cases the chemical throws the weeds into a deep dormancy from which they do not recover when dosage is sufficient.

The mechanism of MH action is not well understood. It has anti-auxin properties and it stimulates destruction of IAA by IAA oxidase; the optimum pH for this is 5.6. Respiration of potato tuber tissue is stimulated by low concentration of MH, inhibited by high.

MH is absorbed rather slowly by plant leaves. Smith (49a) reported 20% of a given dose (1.7 mg.) absorbed in 4 hours; 30% in 8 hours; 60% in 24 hours; and 90% in 48 hours. MH is not activated by acid salts nor is its effectiveness increased by use in an ester form. The most active form seems to be the diethanolamine salt (MH-30 of the Naugatuck Chemical Co.) and absorption is increased by use of surfactants, humectants (i.e., glycerin), and high humidity. These observations seem to indicate migration along an aqueous route, possibly exposed pectin of the leaf surface. MH is highly water soluble and tests with the tagged material indicate that once it is inside the plant it moves very freely compared with 2,4-D which seems to be fairly strongly fixed in tissues.

MH has no primary effect on plants comparable to epicotyl bending in bean and epinasty in tomato. Treated leaves of plants turn a dull green and remain unchanged in appearance for weeks. Apical dominance is repressed and axillary buds are released. Secondary formative effects are: formation of narrow leaves by sugar beet, shoe string virus-like symptoms of tomato, and malformed leaves of cotton. Plants that do not die eventually recover.

## VIII. *N*-1-Naphthyl Phthalamic Acid



(XIX)

The material sold under the name of Alanap-1 (XIX) is a selective preemergence herbicide used for controlling annual broad-leafed weeds and grasses in asparagus, cucurbit crops, and nursery plantings. It comes as a wettable powder and requires agitation in the spray tank. Alanap-3 is the sodium salt of the above acid. It comes as a liquid formulation and requires no agitation after it has been thoroughly mixed in the tank. It is recommended as a pre-emergence or a post-emergence spray on irrigated cotton and on soybeans.

Alanap-2 is *N*-1-naphthyl phthalimide. It is the least soluble of the three compounds. It is recommended for use in potatoes, sweet potatoes, and spinach.

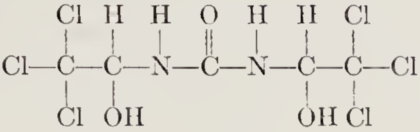
These materials will not kill weeds by contact. They act through the moist soil on the roots of seedling weeds. They are effective against pigweeds, purslane, lamb's-quarters, annual ragweeds, cocklebur, chickweed, annual bindweed, foxtail, crabgrass, watergrass, and similar weeds.

The Alanaps cause dwarfing, leaf malformations, epinastic effects, and inhibition of the geotropic response of roots of growing plants. The main phytotoxic action is on the roots, and the loss of geotropism may indicate an auxin interaction.

Selectivity of the Alanaps is variable; most cucurbit crops are tolerant but a few are injured, particularly when dosage is high enough to control some of the more resistant weeds. A few common annual weeds that are not controlled include dog fennel, buckwheat, sunflower, knotweed, and horse nettle. Bermuda and Johnson grasses are not effectively controlled.

## IX. Substituted Ureas

The first of the substituted ureas to be used for weed control was dichloral urea (XX).

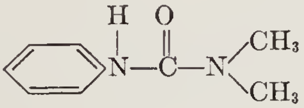


$$CCl_3-CH(OH)-NH-CO-NH-CH(OH)-CCl_3$$

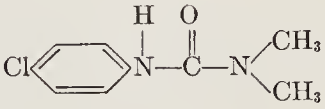
(XX)

It was recommended as a pre-emergence material, toxic to grasses and certain broad-leafed crops. After several years testing it is finding use in sugar beets, cotton, cucurbit crops, and onions. It can be used in the soil against late germinating annual grasses and it has been tested for controlling crabgrass in lawns.

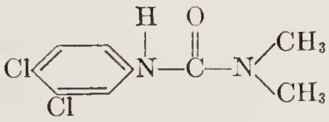
Of tremendous importance at the present time are the asymmetric substituted phenyl ureas. Four of these put out by the Du Pont Company are: 3-(phenyl)-1,1-dimethylurea (XXI), fenuron; 3-(*p*-chlorophenyl) 1,1-dimethylurea (XXIa), monuron; 3-(3,4-dichlorophenyl)-1,1-dimethylurea (XXII), diuron; and 3-(3,4-dichlorophenyl)-1-methyl-1-*n*-butylurea (XXIIa), neburon.



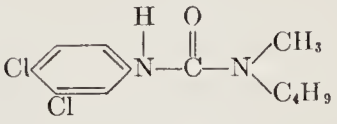
(XXI)



(XXIa)



(XXII)



(XXIIa)

These four compounds are all soil sterilants; they kill plants by root absorption; they are transported upward in the transpiration stream but they are not carried downward in the phloem. They decrease in water solubility in the order given above and their uses are directly related to their solubility. Their phytotoxicity increases in approximately the order presented but there are some selectivities that have to be considered.

Fenuron has a solubility of 2900 p.p.m. at room temperature. It is rather readily leached into soils by rainfall or irrigation water. It is indicated for controlling medium to deep rooted perennial weeds in regions of moderate rainfall and shallow rooted weeds in arid regions.

Monuron has a solubility of 230 p.p.m. at ordinary temperatures. It remains in the top layer of soil and is slowly leached. It is commonly used as an industrial and agricultural soil sterilant for use in average rainfall conditions. It is being tested and shows promise in orchards, vineyards, and caneberries. Where rainfall is high the dichloro compound (Diuron) is used. In low dosages it is used pre- or post-emergence in certain crops such as spinach, cotton, asparagus, grass seed crops, and citrus.

Diuron has a solubility of 42 p.p.m. at ordinary temperatures. Being lower in solubility than the above two compounds it is used for industrial and agricultural soil sterilization in regions of very heavy rainfall. It is used pre-emergence in cotton and other crops and is safer than monuron because of its low solubility and restricted leaching.

Neburon is even lower in solubility being soluble to 4.8 p.p.m. at ordinary temperature. This compound is recommended for crabgrass and chickweed control in lawns and should be applied in spring before the crabgrass seed germinates.

It lays on top of the soil and attacks the first roots that emerge from the crabgrass seeds. It is not soluble enough to leach down to the turf grass roots. Though neburon is not especially toxic against a wide variety of weeds, especially those with deep roots, it has a wide margin of selectivity and where the situation is adapted to its properties it is very effective.

These four urea herbicides are persistent in soils, their breaikdown being accomplished by microorganisms. Toxicity is not directly correlated with clay content nor fertility but seems to be controlled by the types of colloids present. Hill (34a) found for instance that on a bentonite clay 150 p.p.m. were required on the colloid to maintain 1 p.p.m. available in the soil solution. On a kaolinite, on the other hand, less than 1 p.p.m. was required. Quantity and quality of organic matter are also involved in the toxicity of these materials in soils.

Very little is known of the mode of action of these herbicides. Freed (24) postulates that they affect nitrogen metabolism but little evidence is offered for his conclusion. They are readily absorbed from the soil and accumulated in the tops of plants (34). The plants, however, are unaffected for several days whereupon they start dying back from the leaf tips and finally the whole plant succumbs.

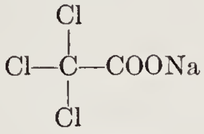
Minshall (36) finds that light has a tendency to intensify the toxic effects on the exposed side of a plant but this may be no more than a localization of concentration through increased transpiration.

A few weeds are resistant to the toxic action of the ureas; one of these is turkey mullein (*Eremocarpus setigerus*). Where it is prevalent, inclusion of 2,4-D acid along with the ureas will handle the situation.

Because of their low solubility these compounds are all marketed as wettable powders and agitation is required in the spray tank. Formulated with borax (Ureabor) monuron is put out in pelleted form. This facilitates dry application by hand spreader, ground rig, or airplane. The boron included in the formulation is reported to reduce biological breakdown of the monuron and hence to prolong its action. Another mixed formulation contains monuron and sodium chlorate (Chlorea). This material combines the rapid killing and ready leaching of chlorate with the persistent sterilizing action of the urea herbicide. A combination of fenuron and monuron or diuron should have similar advantages when both perennial and annual weeds are present.

## X. Trichloroacetic Acid and Dalapon

Trichloroacetic acid (TCA) (XXIII) is the base chemical for a potent grass killer. The sodium salt of this acid:



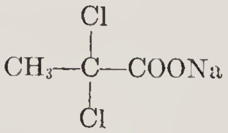
$$\begin{array}{l} \quad\ \ \mathrm{Cl} \\ \quad\ \ | \\ \mathrm{Cl{-}C{-}COONa} \\ \quad\ \ | \\ \quad\ \ \mathrm{Cl} \end{array}$$

(XXIII)

has been used for several years to control grasses in crops and on non-tilled land, i.e., ditchbanks. TCA is a very soluble compound and it is used through the soil. It is readily absorbed by roots and distributed by the transpiration stream; it is not carried downward in the phloem and hence cannot be used as a translocated phytocide against perennial grasses. At rather high dosages in the soil TCA has proved effective for handling Johnson, Bermuda, and quack grasses. At lower dosages it is used against annual grasses in sugar beets, corn, soybeans, etc. By directed spraying it can be used post-emergence against young grasses if subsequent rainfall or irrigation occur to carry it into the soil. TCA has proved unsafe for controlling perennial grasses in orchards and vineyards because almost invariably there is injury to the trees or vines.

Little is known of the action of TCA. The acid is a strong protein precipitant but that can scarcely be concerned in the action of a chemical that is readily absorbed by roots and translocated to the tops where it brings about drastic formative effects. Many of the symptoms on grasses resemble those produced by 2,4-D.

The compound 2,2-dichloropropionic acid (XXIV) reacts with sodium hydroxide to form the propionate (Dalapon):



$$CH_3\text{—}\overset{\displaystyle Cl}{\underset{\displaystyle Cl}{\overset{|}{\underset{|}{C}}}}\text{—}COONa$$

(XXIV)

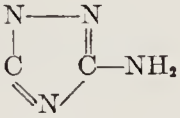
This is a translocated grass killer. It is carried downward in the phloem after application to leaves and upward in the xylem when applied through the soil. It is effective as a foliage spray on perennial grasses (Johnson, Bermuda, quack, etc.) and as a soil treatment against annual or perennial grass seedlings. In contrast to MH, disking or plowing soon after spraying with Dalapon reduces the effectiveness of the herbicide. This suggests that prolonged downward translocation builds up a lethal concentration in the rhizomes.

Dalapon is proving useful for controlling perennial grasses in cotton, corn, sugar beets, sugar cane, orchards and vineyards, and on industrial sites, irrigation canals, roadsides, etc. It has proved very effective against common reed (*Phragmites communis*). It is also useful against young annual grasses by directed spraying in cotton, sugar beets, beans, and other row crops. It is effective against ryegrass, green and yellow foxtail, wild oats, cheatgrass, and ripgut in established alfalfa. It should be applied during winter or immediately after cutting.

Dalapon has drastic formative effects on plants but it seems to lack the stimulative action of the hormone-like materials such as 2,4-D. Experiments prove it to be a noncompetitive inhibitor of straight growth (57) and the suggestion has been made (57) that it might be equivalent to the side chain present on the 2,4-D and IAA molecules. It responds to acid buffering by increase in effectiveness which indicates that it traverses a lipoid barrier in the undissociated form. Since 1,2-dichloropropionic acid and 2,2,3-trichloropropionic acids are less active it seems that the molecular architecture has a profound effect upon its physiological action. Although Dalapon is readily absorbed by roots it seems to cause greatest primary injury to plant tops and translocation studies indicate that it does not move freely from tops to roots. Since grass roots have no buds and hence cannot regenerate this may be a key to the selectivity of this toxicant against grasses.

## XI. Amino Triazole (ATA)

The new translocated herbicide, 3-amino-1,2,4-triazole (XXV), is one of the most interesting of the phytocides available. It is a defoliant, a growth inhibitor, and a physiological regulant causing chlorophyll destruction and impaired chlorophyll synthesis.



(XXV)

The herbicidal properties of ATA have been widely tested. The chemical is absorbed by roots from the soil and transported in the transpiration stream; applied to the leaves it is absorbed and moved downward with food materials through the phloem in the assimilate stream. Visible symptoms of chlorosis appear first on young growing leaves at the stem tips indicating movement to regions of rapid food utilization. Use of labeled ATA shows that movement downward into roots also occurs.

Because ATA is adsorbed by soil colloids and rapidly decomposed in warm moist soils it is not generally recommended as a soil treatment. However, it is tolerated by certain cucurbit crops, cranberries, sugar beets, and corn. It is suggested for pre-emergence treatment in the cucurbits, for pre-planting application in sugar beets, and for post-planting treatment with drop nozzles in corn. Main use of ATA on weeds is as a translocated spray and because the residues in soils do not persist, perennial weeds may be treated and a crop produced later in the season.

Against quack grass ATA should be applied in the spring to young vigorous growth. One to two weeks later, when young chlorotic leaves appear, the area should be plowed or very heavily disked, whereupon the land may be prepared and seeded. Corn, beans, or tomato transplants can be planted in most soils if one week is allowed between spraying and plowing. If two weeks are allowed most vegetable crops may be planted.

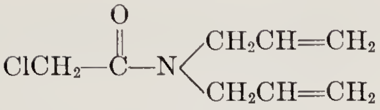
Canada thistle is another weed that is very susceptible to ATA. It need not be plowed after spraying. Other susceptible weeds are milkweed (*Asclepias syriaia*), nut grass (*Cyperus rotundus* and *esculentus*), perennial sow thistle, leafy spurge, toad flax, Russian knapweed, hoary cress, poverty weed, horsetail, and hemp neetle. Under favorable conditions it has controlled Johnson grass, Bermuda grass, cattails, and tules, and a number of grasses including giant and green foxtail. Volunteer alfalfa is readily killed. Woody plants susceptible to ATA are white ash, prickly ash, buckbrush, black oak, hickory, poison oak and ivy. Many other species have been treated and additional names will be added when testing is complete.

Work on the mode of action of ATA is in a preliminary state. In Texas, Hall *et al.* (32) have shown it to be an inhibitor of growth on cotton stems and oat coleoptile sections. The stimulatory effect of IAA on Avena section growth was enhanced by ATA at low concentrations, reduced at high. At relatively high concentration ATA partially relieved the inhibitory action of MH; at higher concentrations MH and ATA in combination were additive in inhibitory action. Competitive inhibition experiments showed that ATA antagonized the effect of IAA in a manner qualitatively but not quantitatively similar to an anti-auxin.

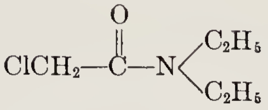
Absorption tests with labeled ATA show that repression of ionization by buffering on the basic side enhances movement into leaves. Once it is inside the plant ATA moves with relative freedom showing much less tendency to be tied up than 2,4-D. It may be very generally distributed in a plant without producing symptoms; young chlorotic leaves of bean had high concentrations of the labeled ATA.

## XII. Alpha-Chloroacetamides

Two new pre-emergence herbicides have been introduced recently by Monsanto, $\alpha$-chloro-*N*,*N*-diallylacetamide (CDAA) (XXVI) and $\alpha$-chloro-*N*,*N*-diethylacetamide (XXVII).



(XXVI)



(XXVII)

These compounds are grass killers with limited selectivity on broad-leafed weeds. With proper dosage they can be used safely on field corn, soybeans, flax, sugar beets, cotton, peanuts, sweet corn, onions, peas, beans, asparagus, potatoes, table beets, turnips, radishes, spinach, and broccoli. They should be useful in sugar cane and sorghum.

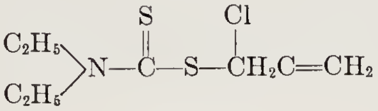
The diallyl compound has proved the most effective in field trials. There must be rainfall or irrigation following application to get them into moist soil where the weed seeds are germinating. Since they have contact action on foliage these materials should not be applied post-emergence on crops.

These materials should not be allowed to come in contact with the skin at full formulation strength as they are irritating. Their action on plant cells is not known but it seems to be largely a contact kill of roots.

It should be recognized that chemicals that are safe to use on so great a variety of crops cannot at the same time control a wide variety of weeds. The chloroacetamides are effective mainly against annual grasses; where mustards, pigweeds, ragweeds, and other broad-leafed annuals abound other materials will be required.

## XIII. Dithiocarbamates

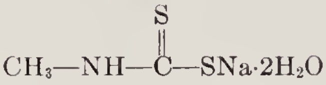
The dithiocarbamate radical has toxicity for both weeds and insects. A recent one, 2-chloroallyl-diethyl-dithiocarbamate(CDEC) (XXVIII), introduced by Monsanto is recommended as a pre-emergence and post-emergence herbicide. It is recommended for pre-emergence use on field corn, soybeans, flax, sugar beets, cotton, peanuts, alfalfa, red clover, ladino clover, birdsfoot trefoil, sweet corn, onions, peas, turnips, spinach, and broccoli. Post-emergence treatment has caused little or no injury on field corn, alfalfa, red



(XXVIII)

clover, ladino clover, and birdsfoot trefoil. CDEC seems better adapted for use on sandy soils, CDAA and CDEA on clay or organic soils.

A dithiocarbamate derivative of an entirely different nature is available from Stauffer. It is sodium *N*-methyl dithiocarbamate dihydrate (XXIX), termed Vapam. This is a water soluble salt which upon contact with moisture undergoes decomposition to form volatile end-products having marked biocidal activity. It is used as a soil fumigant and under proper conditions of soil moisture and application it kills fungi, bacteria, nematodes, insects, and weeds including weed seeds. Vapam is most effective in light-textured soils with a moisture content near field capacity. It is effective against growing rhizomes of Johnson grass, Bermuda grass, quack grass, and similar weeds, against tubers of nutgrass and bulbs of wild onion and garlic.



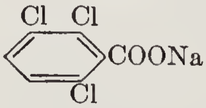
$$CH_3\text{—}NH\text{—}\overset{\overset{S}{\|}}{C}\text{—}SNa\cdot 2H_2O$$

(XXIX)

At proper dosage it will kill deep rooted perennials such as field bindweed and Russian knapweed. It is excellent for seed bed fumigation, spot treatment of perennial weeds, and for field fumigation against nematodes and fungi. It comes as a liquid formulation. For application it is diluted with water to a convenient volume and sprayed or sprinkled over the area to be treated. Application should be followed immediately by sealing either by rolling or by sprinkling with enough water to thoroughly wet the top six inches of soil.

## XIV. Chlorobenzoic Acids

Salts, esters, and emulsifiable acid formulations of 2,3,6-trichlorobenzoic acid, 2,3,5,6-tetrachlorobenzoic acid, and mixtures of these with and without additional isomers are being tested as pre- and post-emergence herbicides.



(XXX)

Apparently 2,3,6-trichlorobenzoic acid (2,3,6-TBA) (XXX), is the most active form of the poly chlorobenzoic acids. This material is a potent growth regulator producing drastic formative effects, similar to, but not identical with those of 2,4-D. The compound 2,3,6-triiodobenzoic acid (TIBA) is a well known anti-auxin, defoliant, and growth regulator. It is difficult to reconcile these facts with the strong formative action of the trichloro compound. Evidently there is a strong auxin interaction that disturbs plant metabolism and upsets differentiation processes.

The benzoic acid herbicides have high initial and residual activity when used as pre-emergence herbicides. Corn and sorghum tolerate them, most other crops and many weeds succumb.

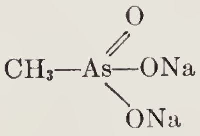
A mixture of trichlorobenzoic isomers consisting mainly of the 2,3,6 form has been widely tested in both water soluble and oil soluble formulations. The former were designed for use as selective sprays in crops much as 2,4-D is used; the latter was designed for basal spraying on woody plants.

In viewing the lists of susceptible, moderately resistant, and resistant weeds that have evolved from the testing of the selective formulation it seems evident that the susceptible ones are the common broad-leafed annuals; the intermediate are shallow rooted perennials and annual grasses; the resistant are nutgrass, bindweed, and horsenettle. This would seem to indicate that this formulation does not translocate well. The chlorobenzoic acids are tolerated by monocotyledonous plants much as the chlorophenoxy compounds are. They seem also to behave in soils much as do the latter.

Two weeds highly sensitive to 2,3,6-TBA are smartweed and milkweed. Susceptible crops are beets, peas, and strawberries.

## XV. Organic Arsenicals

After several years of testing the compound, disodium methyl arsonate (XXXI) has proved a valuable crabgrass killer in turf and lawns.



$$CH_3\text{—}As(=O)(ONa)\text{—}ONa$$

(XXXI)

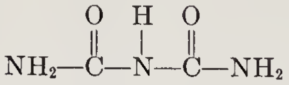
This material is less hazardous to use than sodium arsenite and it is much more selective. In addition to crabgrass, it is effective against watergrass, foxtail, and witchgrass.

Disodium methyl arsonate is available in a liquid formulation containing 2 lb. per gallon, and the directions call for 2 oz. of this formulation in 2 gal. of water to be sprayed over 500 sq. ft. of lawn. Turf grasses and clover will not be killed; the above-mentioned weedy grasses will die if this application is made twice with 5 to 7 days between. If crabgrass continues to germinate, the applications will have to be repeated. For mature crabgrass, barnyard grass, and witch grass, double the above dosage and spray at least twice. The turf should be watered before treatment and again 3 or 4 days after treatment.

In using disodium methyl arsonate the usual precautions required for safe handling of arsenicals should be observed.

## XVI. New Compounds Undergoing Trial

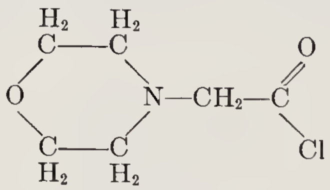
A number of new compounds have been distributed to investigators around the country for testing as herbicides. Most of them have had some screening and testing in the laboratories of the manufacturers; all need thorough trials in the field before they can be recommended for practical use.



$$NH_2-\overset{\overset{O}{\|}}{C}-\overset{\overset{H}{|}}{N}-\overset{\overset{O}{\|}}{C}-NH_2$$

(XXXII)

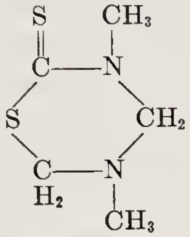
Biuret (XXXII), a nitrogen source as well as a toxicant, has been tested as a combined herbicide and fertilizer in corn (9). It was applied both pre-emergence and post-emergence and at rates of 40 and 60 lb. per acre it effected appreciable weed control, especially against grasses. It resulted in stover yields comparable to those from plots side-dressed with a 10-10-10 fertilizer at 300 lb. per acre.



(XXXIII)

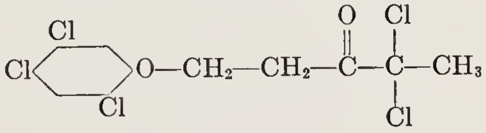
Used as a pre-emergence herbicide 4-(chloroacetyl)morpholine

(XXXIII) offers promise as a weed killer in certain legume crops such as lima, soy and snap beans, and in cotton, corn, and a number of other row crops. It is effective against grasses and has somewhat different selectivities than the α-chloroacetamides. Where broad-leafed weeds abound this chemical will probably require a supplement to give a broad spectrum control.



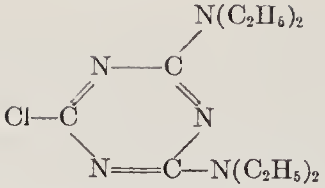
(XXXIV)

Crag Mylone, 3,5-dimethyltetrahydro-1,3,5,2H-thiadiazine-2-thione (XXXIV), is a soil-borne toxicant that acts as a fungicide, nematicide, and herbicide (19). It comes as a wettable powder for application as a dry powder, a water suspension, or mixed with sand. After application it should be sprinkled, or it may be mixed with moist soil by double discing or roto-tilling. It is effective against a great variety of weeds including crabgrass and Bermuda grass.



(XXXV)

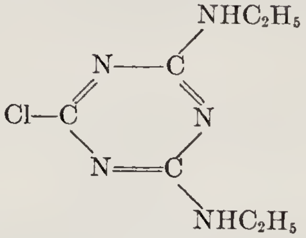
Erbon, 2-(2,4,5-trichlorophenoxy)-ethyl-2,2-dichloropropionate. (XXXV), is the active ingredient in the Dow product Baron, a non-selective herbicide having chronic and systemic effects upon plants as well as soil sterilant properties. It is effective against both broad-leafed plants and grasses but has little or not direct formative action. For this reason it is relatively safe to use around 2,4-D sensitive plants so long as it is not carried as spray drift in large quantities (51). Being persistent in the soil it will probably find its greatest use through the soil. Most weeds are susceptible to erbon and effective kills are reported on Bermuda grass, Johnson grass, quack grass, blue grass, wild carrot, and ragweed; smooth and curled dock seem resistant (22). Like all soil-borne toxicants erbon requires soil moisture to be effective.



(XXXVI)

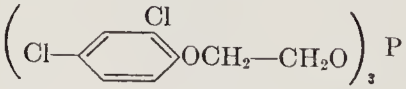
A new product of the Geigy company, 2-chloro-4,6-bis (diethylamino)-*S*-triazine (XXXVI), Geigy 444, is a pre- and post-emergence herbicide. It is insoluble in water but readily soluble in xylene, alcohol, acetone, and chloroform. It comes as a liquid, emulsifiable formulation and makes a milky emulsion in water, a true solution in fuel oil. Used pre-emergence in greenhouse tests it appears to be satisfactory for controlling rye grass, crabgrass, barnyard grass (water grass), and purslane in lima beans, cotton, corn, peas, and soybeans.

In field tests this chemical shows promise in corn, beans, and cotton by both pre-emergence and post-emergence directed spray treatments. In addition to the four weeds mentioned above, chickweed, nut grass, panic grass, ragweed, pigweed, oxalis, plantain, and wild onion have been controlled (28). Additional crops tested are listed in their 1956 technical bulletin (29).



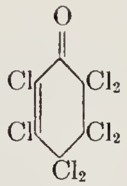
(XXXVII)

Simazin, 2-chloro-4,6-bis (ethylamino)-S-triazine (XXXVII), closely related to Geigy 444 is somewhat more toxic but less selective than the latter. It is reported effective against carpet weed, wild carrot, wild onion, lamb's-quarters, narrow-leafed plantain, purslane, ragweed, and panic grass. Corn is quite tolerant to this chemical, cotton somewhat less so. It comes as a white crystalline powder, insoluble in water, and has been used mainly as a pre-emergence material (30).



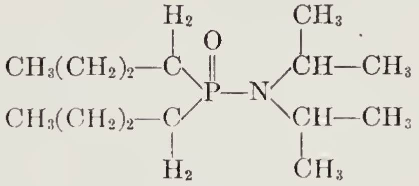
(XXXVIII)

EH3Y9, tris-(2,4-dichlorophenoxy ethyl) phosphite (XXXVIII), of Naugatuck, is a viscous liquid, insoluble in water but completely miscible in benzene, xylene, and other aromatic hydrocarbons. It is formulated as an emulsifiable liquid and is used as a pre-emergence herbicide on most crops against common annual broad-leafed and grassy weeds. It is suggested for pre-emergence treatment of asparagus, corn, onions, peas, soybeans, strawberries, and turf. For post-emergence as a directed spray it is suggested for use on blackberries, blueberries, gladiolus, herbaceous and woody ornamentals, peanuts (lay-by), pepper (after first cultivation), potato (lay-by), raspberries, and established strawberries. Dosages must take into account the textural grade of the soil; the chemical remains active for 3 to 7 weeks (40).



(XXXIX)

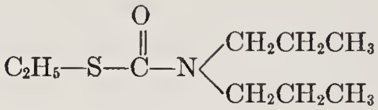
Goodrich Oktone, octochlorocyclohexenone (XXXIX), is a rapid-acting contact toxicant used to fortify diesel oil, kerosene, or fuel oil. It is general in its action and it breaks down rapidly in the soil. It is recommended that Oktone be used against small weeds as they require a minimum volume of the spray solution. Oktone can be used pre-emergence against weeds in asparagus, rhubarb, and peony (31).



(XL)

The organic phosphorus compound *P,P*-dibutyl-*N,N*-diisopropylphosphinic amide (XL), Shell SD 1369, is a new herbicide, defoliant, and plant desiccant produced by the Shell Development Co. It is a contact toxicant affecting the chlorophyll mechanism of the green cells and it has some anti-auxin action. It has a very low solubility in water, medium solubility in aliphatic oils, and high solubility in aromatic solvents and acetone. It is available as an emulsifiable liquid concentrate.

After application SD 1369 causes bleaching and drying of tissues in the presence of light. Applied to the soil it is absorbed by roots, translocated through the xylem, and causes bleaching and death of the foliage of seedlings. It is effective against all weeds but is somewhat more toxic to broad-leafed plants than to grasses. It also has algicidal action, being more rapid in its action than copper sulfate and nontoxic to fish (48).



(XLI)

Stauffer EPTC, ethyl *N*,*N*-di-*n*-propylthiolcarbamate (XLI) is a new pre- and post-emergence herbicide tested on forage legumes and certain vegetable crops. Corn, tomatoes, carrots, and beets have been found to be resistant to pre-emergence application. Most crops have proved resistant to post-emergence use. In pre-emergence tests it is effective against grasses, pigweed, and purslane. EPTC kills weed seeds to some depth in the soil when leached in with moving soil moisture.

This final group of compounds represents a large amount of synthesis, screening, and testing on the part of industry. As one views them it is apparent that they represent greater and greater complexity of molecular configuration. This may evidence two possible trends: (1) a search for, and development of greater selectivity, or (2) a tendency to put out compounds of unique chemical composition that can be patented. If selectivity is the objective of the tremendous efforts being expended by industry, it is commendable because we need herbicides to fit into many niches not filled at the present time. Less expensive and less harmful pre-emergence chemicals are needed in corn and sorghum crops. More selective materials are required for use in soybeans, field beans, and snap beans. A general pre-emergence spray for control of both broad-leafed weeds and grasses is needed for cotton. And highly selective materials are desirable in sugar beets, tomatoes, and a host of vegetable crops.

If the main objective of synthesis and testing of herbicides is the discovery of patentable chemicals it is unfortunate because a good many of the basic groups are already covered and the remaining toxicants of merit are almost bound to be more complex. Since our knowledge of biochemistry of herbicide toxicity is limited, the continuing search is bound to be highly empirical and increasingly costly. Undoubtedly it will eventually reach a point of diminishing returns and industry will probably withdraw from the field. When this occurs it will be necessary for university, state, and federal groups to reenter the field and proceed with the search. What does the future hold for such research? Certainly the trend is toward more specific weed killers, more effective herbicides to fit the many niches that still require filling. We need better materials to aid in reclaiming abused range lands, to aid in reforestation, to improve pastures and turf, to take the drudge work out of farming, and to make gardening more pleasant.

Our research programs will need more time, they must use more crops, more weeds, and a wider variety of conditions. Screening will have to be aimed at the special problems and this requires that the technicians have more training and more familiarity with field situations. Meanwhile the compounds described above, and many others still in the screening process, will require exhaustive testing and study. Fundamental research on mechanisms of selectivity of herbicides are needed to reduce as much as possible the great amount of work now required to find and prove a new chemical. Progress cannot continue if costs of synthesis and screening continue to spiral as they have during the past decade.

## XVII. Discussion

When we take an over-all look at the chemistry and mode of action of chemical weed killers it is evident that we have very little basic information upon which, for instance, one might base a search for new and better materials. We have a large volume of empirical data on selectivities, dosages, and application requirements, based on greenhouse and field screening. In many cases such work tells us much concerning the possibilities and limitations of a chemical. Often much information on the physiology and hence the essential requirements for proper application may be gleaned. But when it comes to the ultimate biochemistry of mechanism at the subcellular level little or nothing is known.

Going back to the older materials, arsenic is known to uncouple the energy storing triosephosphate oxidation of the respiration process in cells. In strongly acid or alkaline solution it is rapidly absorbed, probably by altering the permeability of the leaf surface. It is translocated only in the xylem and in the vital tissues it is a strong protein coagulant. In any form it has violent contact action.

Chlorate is known as a strong oxidizing agent; it is absorbed from high concentrations in leaves, translocated through xylem, and brings about a slower, more subtle action against the protoplasm. It is also absorbed from the soil by roots, strongly in the absence of nitrates, less in their presence. Åberg (1) suggests that chlorate toxicity results from reduction of the chlorate to hypochlorates or chlorites by the mechanism that normally reduces nitrate.

Borates disturb the normal function of plant pigments. Boron toxicity is accompanied by a certain degree of chlorosis.

Borates have contact action on foliage if applied in high concentration; they are usually strongly alkaline in their reaction. In the soil they must be present at relatively high concentrations to kill plants. They are translocated in the xylem and their action at the ultimate sites results from contact kill through accumulation.

The dinitro-phenols are known for their role in uncoupling the oxidative phosphorylation of carbohydrates in the respiration mechanism. They were the first herbicides to act at low dosage rates. They are absorbed best as undissociated molecules through the lipoid phase of the plant surface; they are translocated only in the xylem; they have strong contact action at the ultimate site of cell response.

When we tackle the chlorophenoxy compounds we are on much less certain ground. We know that they have auxin-like properties but that is of little help because we do not yet understand the mechanism of auxin action. Furthermore the total response to 2,4-D involves more than a growth response; toxicity of a severe type is involved and death of cells is the ultimate aim of its use.

Auxin response involves primarily relaxation and growth of cell walls. The two-point attachment hypothesis or some such mechanism is probably involved in this response and it offers through the Lineweaver-Burk analysis a nice rationalization for the normal stimulation-inhibition curve found when the reciprocal of growth is plotted against the reciprocal of auxin concentration. However, this explains only stimulation to an optimum point and then reversal of this process back to normal growth as concentration is increased.

Nothing is told of the reaction responsible for the drastic formative effects observed and injury and death are completely outside the picture.

Other types of auxin study tell us whether an unknown chemical is an auxin, an anti-auxin, a hemi-auxin, or a coenzyme in the auxin response mechanism. Again formative and lethal effects are not explained.

Physiological studies indicate that 2,4-D is best absorbed in the undissociated form across a lipoid barrier; it migrates rapidly across the living mesophyll; it enters the phloem and moves with the assimilate stream to regions of food utilization; it probably accumulates in meristems and brings about dramatic changes in differentiation processes and if present in sufficient amounts, ultimate death. Studies (8) have shown that 2,4-D will leak from roots into the culture solution following its application to foliage. We also know that it is absorbed from the culture medium by roots and transported in the transpiration stream. From these two observations it should be obvious that it will be released from the phloem into the xylem and hence will circulate in the plant in the same way that phosphorus does. However, we also know that 2,4-D is metabolized in the plant and that the carbon is released as $CO_2$. In this way its end products may enter the assimilate pool and contribute to the organic nutrition of the plant. This undoubtedly represents only a passive phase of its role in plants, probably unrelated to its lethal action.

Isopropylphenylcarbamate is known to be a mitotic poison in plants and its use as a soil-borne toxicant is related to this fact. It has little or no contact action on foliage and must be applied through the soil. This latter relation is a boone in a way in that it allows application of the chemical as a wettable powder or in pelleted form for control of weed seedlings in a growing crop such as alfalfa or clover. In other crops it may be applied pre- or post-emergence for controlling germinating seedlings. It is rather highly selective against grasses and a few broad-leafed weeds.

Ammonium sulfamate and maleic hydrazide are known to be growth inhibitors, the former used at high concentrations has drastic contact action followed by a strong induction of dormancy; MH is used in smaller amounts and it induces dormancy without necessarily causing a contact burn. MH is known to have anti-auxin properties and strong auxin interaction.

This about exhausts our present knowledge of the mode of herbicide action. Few of the newer materials have received sufficient study to give more than empirical information. Much more needs to be known of the physiology of these interesting materials if we are going to be able to use them to the greatest advantage. More of their biochemistry is needed if we are going to have the knowledge necessary for an intelligent search for more and better herbicides. And an immense body of biological information will be required before we will be able to fit each into its proper niche in the over-all scheme of chemical weed control.

## References

1. Åberg, B., *Ann. Roy. Agr. Coll. Sweden, 15,* 37 (1947).
2. Allard, R. W., Ennis, W. B., De Rose, H. R., and Weaver, R. J., *Botan. Gaz., 107,* 589 (1946).
3. Anderson, J. C., and Ahlgren, G., *Down to Earth, 3,* No. 1, 16 (1947).
4. Anderson, J. C., and Ahlgren, G., *Down to Earth, 3,* No. 4, 13 (1948).
5. Anderson, J. C., and Wolf, D. E., *J. Am. Soc. Agron., 39,* 341 (1947).
6. Bohmont, D., *Western Weed Control Conf. Research Progress Report, 13,* 12 (1952).
7. Carlson, R. F., *Mich. Agr. Expt. Sta. Quart. Bull., 29,* 274 (1947).
8. Clor, M., M.S. Thesis, Univ. of Calif., Davis, 1951.
9. Collins, H. A., and Aldrich, R. J., *Northeastern Weed Control Conf. Proc. 10,* 1 (1956).
10. Crafts, A. S., *Science, 101,* 417 (1945).
11. Crafts, A. S., *Science, 108,* 85 (1948).
12. Crafts, A. S., *Western Weed Control Conf. Proc., 12,* 61 (1950).
13. Crafts, A. S., *Botan. Rev., 17,* No. 4, 203 (1951).
14. Crafts, A. S., *Western Weed Control Conf. Proc., 13,* 4 (1952).
15. Crafts, A. S., *Ann. Rev. Plant Physiol., 4,* 253 (1953).
16. Crafts, A. S., *Hilgardia, 26,* No. 6, 287 (1956a).
17. Crafts, A. S., *Hilgardia, 26,* No. 6, 335 (1956b).
18. Crafts, A. S., and Reiber, H. G., *Hilgardia, 16,* No. 10, 487 (1945).
19. Crag Brand 974. Technical information lithograph. Union Carbide and Carbon Chemicals Co., Feb., 1956.
20. Currier, H. B., *Hilgardia, 20,* No. 19, 383 (1951).
21. Currier, H. B., and Peoples, S. A., *Hilgardia, 23,* No. 6, 155 (1954).
22. Dow Chem. Co. Tech. Bull. #1 Erbon. The Dow Chem. Co., Jan., 1955.
23. Ennis, W. B., *Science, 105,* 95 (1947).
24. Freed, V. H., Symposium Rept. Am. Chem. Soc. 122nd Meeting Atlantic City, N. J., 1952.
25. Freed, V. H., *Weeds, 1,* No. 1, 48 (1951).
26. Freed, V. H., and Remmert, L. F., W-11 *Tech. Comm. Ann. Progress Rept.,* Sacramento, Calif., 1956.

27. Gast, R., and Early, J., *Agr. Chemicals, 11*, No. 4, 42 (1956).
28. Geigy, Agr., Chem. Tech. Bull. 55-1 Feb., 1955.
29. Geigy, Agr., Chem. Tech. Bull. 56-1 March, 1956.
30. Geigy, Agr., Chem. Tech. Bull. 56-2 March, 1956.
31. Good-rite Chem., B. F. Goodrich Chem. Co. Service Bull. GA-13, July, 1954
32. Hall, W. C., Johnson, S. P., and Leinweber, C. L., *Texas Agr. Expt. Sta., Bull. 789* (1954).
33. Hamner, C. L., and Tukey, H. B., *Science, 100*, 154 (1944).
34. Haun, J. R., and Peterson, J. H., *Weeds, 3*, No. 2, 177 (1954).
34a. Hill, G. D., Rept. Sect. IV, Charter Meeting Weed Soc. Amer., N. Y., 1956.
35. Ivens, G. W., and Blackman, G. E., *Symposia Soc. Exptl. Biol., 3*, 266 (1949).
35a. Kaufman, P. B., *Weeds, 2*, No. 4, 223 (1953).
36. Minshall, W. H., Report before the Gordon Conf. on Plant Biochem. and Agr. Meriden, N. H., July 1955.
37. Mitchell, J. W., and Marth, P. C., *Science, 106*, 15 (1947).
38. Muir, R. M., and Hansch, C. H., *Ann. Rev. Plant Physiol., 6*, 157 (1955).
39. Muir, R. M., Hansch, C. H., and Gallup, A. H., *Plant Physiol., 24*, 359 (1949).
40. Naugatuck Chem. Div., Bethany Information Sheet No. 83, 1956.
41. Neeley, W. B., Ball, C. D., Hamner, C. L., and Sell, H. M., *Plant Physiol., 25*, 525 (1950).
42. Newman, A. S., and De Rigo, H. T., *Weeds, 2*, No. 3, 169 (1953).
43. Ordin, L., and Bonner, J., Private correspondence (1955).
44. Ordin, L., Applewhite, T. H., and Bonner, J., Private correspondence (1955). This work reported before A.I.B.S. on Sept. 7, 1955, at East Lansing, Mich.
45. Overbeek, J. van., and Blondeau, R., *Weeds, 3*, No. 1, 55 (1954).
46. Robbins, W. W., Crafts, A. S., and Raynor, R. N., *Weed Control*, McGraw-Hill Book Co., New York, N. Y., 1942.
47. Shaw, W. S., and Swanson, C. R., *Weeds, 2*, No. 1, 43 (1953).
48. Shell Dev. Co., Sheet ARD-55-6, June, 1955.
49. Slade, R. E., Templeman, W. G., and Sexton, W. A., *Nature, 155*, 497 (1945).
49a. Smith, A. E., Naugatuck Chem. Div., U. S. Rubber Co. Literature Summary on M. H., MHIS No. 6c., May, 1955.
50. Stone, J. D., and Rake, D. W., *Agr. Chemicals, 10*, No. 5, 36 (1955).
51. Swezey, A. W., *Down to Earth, 11*, No. 3, 10 (1955).
52. Synerholm, M. E., and Zimmerman, P. W., *Contribs. Boyce Thompson Inst., 14*, 369 (1947).
53. Templeman, W. G., and Sexton, W. A., *Nature, 156*, 630 (1945).
54. Veldstra, H., *Ann. Rev. Plant Physiol., 4*, 151 (1953).
55. Wain, R. L., *Agr. and Food Chem., 3*, No. 2, 128 (1955).
56. Westgate, W. A., and Raynor, R. N., *Calif. Agr. Expt. Sta., Bull., 634*, 1 (1940).
57. Wilkinson, R. E., Ph.D. Dissertation, Univ. of Calif., Davis, June, 1956.

# USES OF RADIOISOTOPES IN PESTICIDE RESEARCH[1]

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## I. Introduction

The major accomplishments in applying radiobiology and radiochemistry to pesticide research have been made in the last decade. The results can be divided into two major fields, namely, use of ionizing radiation, and the employment of "tracers" to detect their subsequent action or movements. This review attempts to correlate the uses of radioisotopes in pesticide research with the present state of knowledge in this field. Information relating to radioisotopes and their use in biology and agriculture is available from many sources (17,20,42,59,66,67,86,97,100,141,166,195,264,307). The U. S. Atomic Energy Commission has prepared an eight-year summary of the distribution and uses of isotopes which includes many references to pesticide research (293). More specialized reviews have dealt with

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such areas as entomology (72,73,158,184), epidemiology (156,157), fungicides (167,196,213,214), and the plant sciences (286).

The topics included in this review are: insecticides (DDT and related chemicals, lindane and BHC, organic phosphorus insecticides, pyrethroids and piperonyl butoxide, nicotine, fumigants, and miscellaneous insecticidal chemicals), fungicides, herbicides and plant growth regulators, tagging insects, mites, and ticks with radioisotopes, epidemiology and public health, and food preservation and pest control through radiation effects. Special equipment and techniques are mentioned only if they have some bearing on the pest control problem.

## II. Insecticides

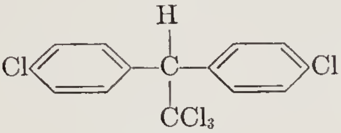
The many modern organic insecticides created by chemists and nurtured through screening tests and field trials by entomologists have provided a fertile field for the use of labeled compounds (73). The enactment in the United States of the Pesticide Chemicals Amendment (P.L. 518, 83rd Congress) to the Federal Food, Drug and Cosmetic Act has not only demonstrated the need for additional evaluation of insecticidal residues on food, feed, and forage but has required more precise information on the metabolism of these chemicals. Most of the significant research with labeled insecticides is reviewed in the following sections.

### A. DDT AND RELATED CHEMICALS

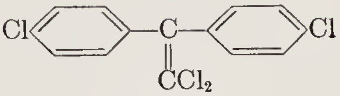
Radioactive DDT and analogues of DDT have been used to study the absorption, internal distribution, and metabolism of the insecticide in insects, chickens, and mammals. Although it has been possible to establish rather precisely the quantitative aspects of DDT metabolism, efforts to discover the exact locus of DDT action and identify the metabolites of DDT in excreta have been less successful. Radioactive DDT has also been very useful in studying the physiological differences between susceptible and DDT-resistant strains of insects.

*Absorption, Metabolism, and Detoxication.* Lindquist *et al.* (187) studied the penetration and metabolism of $C^{14}$-labeled DDT, 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane (I), by applying microdosages to DDT-resistant adult, female houseflies, *Musca domestica*, and by allowing flies to walk over a residue of the insecticide. About

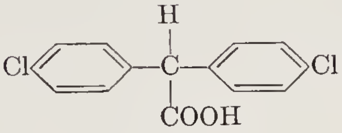
78% of the DDT applied to the flies surviving the topical treatment was recovered. Of the DDT recovered, 18% apparently had penetrated into the body of the fly. Bioassays of the fly extracts showed that 63% of the total DDT absorbed by the surviving, topically treated flies was nontoxic and therefore metabolized. The 27% that was toxic was probably DDT as the two possible metabolites of DDT, designated as DDE, 2,2-bis-(*p*-chlorophenyl)-1,1-dichloroethylene (II), and DDA, 2,2-bis-(*p*-chlorophenyl) acetic acid (III), did not kill the mosquito larvae. In dead susceptible and resistant flies about the same percentage of DDT was metabolized to nontoxic products.



DDT (I)



DDE (II)



DDA (III)

Hoffman *et al.* (144) found that survival increased when a group of DDT-resistant houseflies was allowed longer rest periods between intermittent exposures to small amounts of $C^{14}$-labeled DDT residues. The amounts of DDT absorbed were similar in both cases. Since the mortality of both DDT-resistant and susceptible flies decreased as the time between exposures was lengthened, it was concluded that the flies were able to detoxify the poison. The absorption of DDT apparently proceeds at about the same rate in dead as in live flies (145).

Lindquist *et al.* (186) determined the distribution of $C^{14}$-labeled DDT in adult, female, DDT-resistant houseflies. Approximately 30% of the DDT applied topically was found in the internal organs and the remainder was distributed throughout the cuticle. Flies exposed to a residual deposit of DDT showed a similar distribution of the toxicant. Roth *et al.* (251) found that resistant houseflies individually treated with $C^{14}$-labeled DDT absorbed 64% more of the toxicant with a lower mortality during a 24-hour period at 90°F.

than at 70°. Approximately 15% of the total DDT absorbed was accounted for as radioactivity in the excrement over a 7-day period.

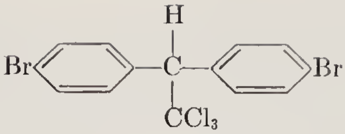
LeRoux and Morrison (182) used the $C^{14}$-labeled DDT prepared by MacDonald and Baker (199) to determine the amount and location of DDT needed to kill a housefly. The mortality of flies treated on their legs with 2 μg. of DDT was reduced by amputation of the treated leg after 30 minutes, but not after 4 hours. Amputation of similarly treated labellae after 30 minutes had no effect. Absorption and distribution of less than 20% of the applied dosage produced the full DDT effect. Thus, the locus of DDT application did influence the insecticide's effectiveness. The same authors (219) suggested that the head of the housefly appears to be a site for the lethal action of DDT. In a later report, LeRoux and Morrison (183) concluded that some organ or product in or produced within the head of the fly appeared to be the site of action of DDT. The locus of topical application of DDT greatly influenced the rate of penetration, distribution, and accumulation at the site of action. Distribution of DDT from the point of application to other parts of the insect body was by way of the hemolymph. A DDT-resistant strain of houseflies absorbed externally applied DDT from all tested loci much more slowly than did the susceptible strain employed.

Perry *et al.* (232) carefully studied the degradation products of DDT in 7 DDT-resistant strains of houseflies, using $C^{14}$-labeled DDT (228,229). The only significant product of DDT metabolism was DDE. Both DDT and DDE were found in the ether-soluble portion of the excreta, the ratio of DDE to DDT increased with time. Very small amounts of a radioactive product were found in the water-soluble portion of the excreta. No strain specificity was evident. Resistant and susceptible houseflies have been examined by Terriere and Schonbrod (279) for evidence of metabolism of DDT after topical treatment with $C^{14}$-tertiary carbon-labeled DDT (56). Flies from both the susceptible strain and the resistant strain excreted up to 88% of the applied DDT in the form of a water-soluble conjugate. The metabolic conjugate was hydrolyzable with acid to produce a compound weakly acidic in nature.

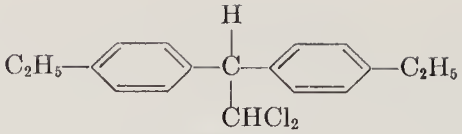
Butts *et al.* (58), using 2-$C^{14}$-ethane-labeled DDT, reported a detoxication product in American cockroaches, *Periplaneta americana*, injected with the insecticide and speculated that the water-soluble, radioactive product was probably a conjugated compound composed of a derivative of DDT and another fragment, possibly carbohydrate in nature. Robbins and Dahm (245) studied the absorption, excretion, tissue distribution, and metabolism of both $p,p'$-$C^{14}$- and 2-$C^{14}$-ethane-labeled DDT and the tissue excretion and distribution of $p,p'$-$C^{14}$-labeled DDE in female, American cockroaches. Topically applied DDT and DDE were rapidly absorbed and widely distributed internally. As much as 75% of the DDT was excreted as metabolites in the feces over a 24-day period. About 80% of the radioactivity in the feces was due to metabolites containing the diphenyl-2-carbon moiety of DDT; less than 10% was due to DDT, DDE, or DDA. When piperonyl cyclonene was used with DDT, it inhibited absorption of DDT and excretion of metabolites. The absorption, metabolism, and excretion of $p,p'$-$C^{14}$-labeled DDT in adult, female Madeira roaches, *Leucophaea maderae*, and 5th instar European corn borer larvae, *Pyrausta nubilalis*, were studied by Lindquist and Dahm (189). The Madeira roach absorbed DDT rather slowly and excreted 50% of the applied radioactivity over a period of 36 days. DDT, DDE, and three metabolites were identified in the feces by paper chromatography. DDT was the predominant radioactive compound excreted the first 24 hours after treatment, after which an unidentified metabolite was the major radioactive compound excreted. Several metabolic pathways for DDT in the Madeira roach have been proposed. Fifth instar European corn borer larvae possess some tolerance to DDT and convert significant amounts of absorbed DDT to DDE. No evidence of other DDT metabolites was found.

Gjullin *et al.* (113), using $C^{14}$-labeled DDT, determined that resistant *Aedes nigromaculis* mosquito larvae absorbed over six times as much DDT as susceptible larvae and both larval strains apparently degraded large amounts of DDT to nontoxic substances.

Hansen *et al.* (128) topically applied the $Br^{82}$ analogue of DDT, 2,2-bis-($p$-bromophenyl)-1,1,1-trichloroethane (IV) and hereinafter referred to as $DBr^{82}DT$, to a number of insects, including adult American cockroaches and larvae of the cadelle, *Tenebroides mauritanicus*, the yellow mealworm, *Tenebrio molitor*, and the wax moth, *Galleria mellonella*. Except for a slightly lower toxicity, $DBr^{82}DT$ behaves like DDT. Autoradiograms indicated the radioisotope was present in the nerve cord and brain and in other parts of the insects.



DBr$^{82}$DT (IV)



Perthane® (V)

Winteringham *et al.* (314) sprayed DBr$^{82}$DT onto wheat which was subsequently milled, baked, and fed to animals. The wheat, carrying 40 p.p.m. of the insecticide, was milled under typical conditions; about 15 p.p.m. was found in the flour and 11 p.p.m. in the bread made from the flour. Some of the grain and bread were fed to chickens and rats. The insecticide was found in all the tissues examined. A fraction of the radioactivity found in the excreta behaved as the 2,2-bis-(*p*-bromophenyl) acetic acid metabolite, hereinafter referred to as DBr$^{82}$DA. This metabolite also was identified in the urine of a man following ingestion of some of the bread. Winteringham *et al.* (309,316) used DBr$^{82}$DT and paper chromatographic methods (308) to study the resistance of houseflies to DDT. DDT-resistant flies were also resistant to DBr$^{82}$DT but their resistance was not associated with decreased absorption of the insecticide. Metabolism of DBr$^{82}$DT was observed only in DDT-resistant houseflies and DBr$^{82}$-DE, 2,2-bis-(*p*-bromophenyl)-1,1-dichloroethylene, was the only metabolite found. There was no evidence of DBr$^{82}$DA or any other metabolite sufficiently different to distinguish it from either DBr$^{82}$DT or DBr$^{82}$DE. These results suggested that DDT metabolism was enzymatic in nature, a conclusion that was subsequently established by Sternburg *et al.* (275). The rate of metabolism appeared to be insufficient to account entirely for the successful resistance of the flies. Only a small fraction of the insecticide seemed to be metabolized at the site of action. To check whether the presence of DBr$^{82}$-DE might account for the observed resistance, mixtures of DBr$^{82}$DT and DBr$^{82}$DE were injected into or applied to susceptible flies. No evidence of protection was observed. Winteringham (305) treated two resistant (from Italy and Sardinia) and two susceptible (from Italy and England) strains of housefly adults and larvae with DBr$^{82}$-DT to study the metabolism of this compound. Both strains were able to metabolize the compound provided the dose absorbed was sufficiently low; the resistant strains degraded the insecticide more rapidly than the susceptible strains did, however. Only the intact, living flies were capable of metabolically degrading the absorbed insecticide. At least two kinds of DDT-resistance were observed, one represented by the Italian strain in which survival appeared to depend upon a mechanism such as enzymatic dehydrohalogenation (275) and the enhanced metabolism was a consequence rather than a cause of survival. In the Sardinian strain resistance did not appear to depend upon such a mechanism.

*Persistence in Soils.* Jackson and Hopkins (150) studied the persistence of $p,p'$-$C^{14}$-labeled DDT in 18 representative soils at an application rate corresponding to 5 lb. of DDT per acre. After storage periods of 1 and 6 months, the soils were extracted with acetone and the amount of radioactivity recovered from each soil was measured. Identification of the degradation products of DDT was made on soil extracts by means of paper partition chromatography (308). Decomposition of the DDT was generally greatest in soils with a pH higher than 7.0. DDE was the principal degradation product in 16 soils. DDA was not persistent in any of the 18 soils.

*Syntheses and Analytical Procedures.* Fields *et al.* (91) have prepared $p,p'$-$C^{14}$-labeled DDT and Pearce and Jensen (228,229), Buhler *et al.* (56), and MacDonald and Baker (199) have synthesized tertiary-$C^{14}$-labeled DDT, 2,2-bis-(*p*-chlorophenyl)-1,1,1-trichloroethane -2-$C^{14}$. Hansen *et al.* (128) and Winteringham *et al.* (314) have synthesized the $Br^{82}$ analogue of DDT, 2,2-bis-(*p*-bromophenyl)-1,1,1-trichloroethane, and Jensen and Pearce (162) have prepared the $I^{131}$ analogue of DDT, 2,2-bis-(*p*-iodophenyl)-1,1,1-trichloroethane.

Winteringham *et al.* (308) used the $DBr^{82}DT$ analogue of DDT in developing a reversed-phase, paper partition chromatographic analytical method for this compound and two possible metabolites, $DBr^{82}DE$ and $DBr^{82}DA$ (309,310).

*Perthane®.* Brandes (46) has reported fat storage studies of $C^{14}$-labeled Perthane®, 1,1-dichloro-2,2-bis-(4′-ethylphenyl-4′-$C^{14}$) ethane (V), in rats. Six male and 6 female albino rats were placed on a diet containing 50 p.p.m. of the chemical for a period of 6 weeks. At the end of this period, the maximum Perthane® concentration in the fat was approximately 19 p.p.m.

## B. LINDANE AND BHC

*Metabolism and Uptake in Insects and Plants.* Bradbury *et al.* (43) and Bradbury and Standen (44) studied the fate of $C^{14}$-labeled γ-benzene hexachloride (142), 1,2,3,4,5,6-hexachlorocyclohexane or BHC (benzene hexachloride), in normal and resistant houseflies. Some of the benzene hexachloride absorbed by both susceptible and resistant strains of flies was converted into water-soluble compounds which were not extracted by carbon tetrachloride. The greater amount of these water-soluble products in resistant flies did not offer sufficient explanation for resistance to the insecticide.

The absorption and metabolism of $C^{14}$-labeled γ-BHC by wheat seedlings grown in an aqueous medium containing the labeled insecticide was measured by Bradbury and Whitaker (45). The seedlings removed increasing amounts of BHC from the culture medium for about 9 days after which a decreasing trend became apparent. These studies suggested that virtually all the insecticide applied as a seed treatment may be absorbed and the main effects of such a treatment are systemic.

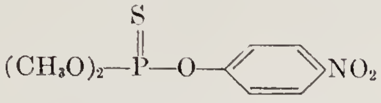
*Synthesis and Analytical Procedures.* Trenner *et al.* (289) developed a mass isotope dilution method for the determination of lindane (the γ-isomer of BHC) and, in principle, for some of the other isomers of BHC in the crude benzene chlorination products. The γ-isomer of hexachlorodeuterobenzene, produced by photochlorination of deuterobenzene, was used as the tracer molecule and the extent of isotopic dilution in the isolated gamma-isomer mixture was determined by means of infrared spectrophotometry. Winteringham *et al.* (309,310) have chromatographed a mixture of α, β, γ, and δ isomers of BHC under the conditions used for separating $DBr^{82}DT$ and metabolites (308). The chromatogram was irradiated at a flux of $10^{11}$ neutrons/cm.$^2$/sec. for one week and scanned two weeks later. The peaks of radioactivity were due to $S^{35}$ arising from the reaction:

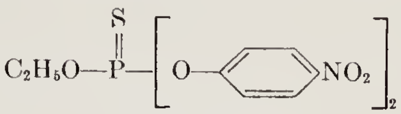
$$Cl^{35} \text{ (from the partly separated BHC isomers) } \xrightarrow{n,p} S^{35}$$

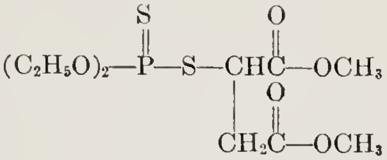
Craig *et al.* (70) developed a $Cl^{36}$ isotope dilution method for determining the γ-isomer content of technical BHC. The standard deviation of the method was ±0.2% in γ-isomer content. Hill *et al.* (142) and Palin (226) have reported the same $C^{14}$-isotope dilution method for determining lindane in crude BHC.

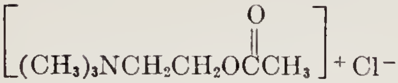
## C. ORGANIC PHOSPHORUS INSECTICIDES

The chemicals in this section can be differentiated on the basis of whether they are largely effective per se or undergo oxidative conversions in plants and animals. All are inhibitors of cholinesterases; their potency depends not only upon their intrinsic enzyme affinity but also on anticholinesterase properties acquired through *in vivo* metabolism. Without radioisotopes, it is doubtful if the information that follows could ever have been accumulated in so short a time.


Methyl parathion (VI)

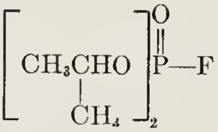

NIUIF-101 (VII)


Carbophos IV (VIII)



$$\left[(CH_3)_3NCH_2CH_2O\overset{\overset{O}{\|}}{C}CH_3\right]^+Cl^-$$

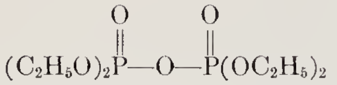
Acetylcholine chloride (IX)



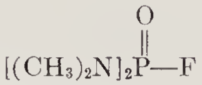
$$\left[\underset{\underset{CH_3}{|}}{CH_3CHO}\right]_2\overset{\overset{O}{\|}}{P}-F$$

Di*iso*propyl fluorophosphate (X)

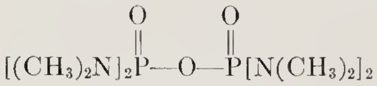
Ripper *et al.* (242) reviewed the translocation and activation of systemic insecticides in plants, including the use of $P^{32}$-labeled schradan (XIII). Wedding (296) has included data from studies with $P^{32}$-labeled schradan and $S^{35}$-labeled demeton (XIX, XXIII) in discussing the permeability, and translocation and activation by plants of systemic insecticides. Metcalf (206) has reviewed some of the advantages of employing radiotracers in studying the mode of action and residues of schradan and demeton, and Heath (136) has reviewed some applications of $P^{32}$ to the study of systemic insecticides. Gar and Kipiani (108) have summarized Russian research with several radioactive organic phosphorus insecticides including: $P^{32}$- and $S^{35}$-labeled parathion (XVII), methyl parathion or *O,O*-dimethyl *O*-*p*-nitrophenyl thiophosphate (VI), NIUIF-101 or *O*-ethyl *O,O*-di-(*p*-nitrophenyl)-thiophosphonate (VII), $P^{32}$-labeled malathion (XXX), and carbophos IV or *O,O*-diethyl-*S*-(1,2-dicarbethoxymethyl) dithiophosphate (VIII). The photochemical stability of formulations of NIUIF-101 and carbophos IV was greater than that of parathion and malathion. Fromulations of parathion and malathion were about equal in their stability to heat and NIUIF-101 and carbophos IV were approximately the same; the latter two were slightly more stable than the former. The plant systemic properties of parathion and methyl parathion were slight; hydrolysis of both compounds proceeds rapidly inside plants. Paper chromatographic studies of alkaline hydrolysates and extracts from plants treated with methyl parathion revealed similar decomposition products; there was no evidence of oxidation and upgrading of toxicity of the insecticide by plants. It was recommended that several of these insecticides could be used on plants shortly before harvesting.


$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}-O-\overset{\overset{O}{\|}}{P}(OC_2H_5)_2$$

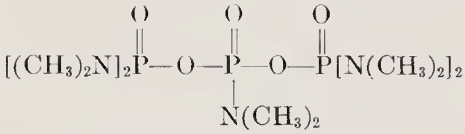
TEPP or tetraethylpyrophosphate (XI)


$$[(CH_3)_2N]_2\overset{\overset{O}{\|}}{P}-F$$

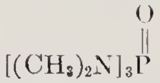
Dimefox (XII)


$$[(CH_3)_2N]_2\overset{\overset{O}{\|}}{P}-O-\overset{\overset{O}{\|}}{P}[N(CH_3)_2]_2$$

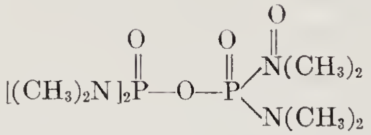
Schradan (XIII)


$$[(CH_3)_2N]_2\overset{\overset{O}{\|}}{P}-O-\underset{\underset{N(CH_3)_2}{|}}{\overset{\overset{O}{\|}}{P}}-O-\overset{\overset{O}{\|}}{P}[N(CH_3)_2]_2$$

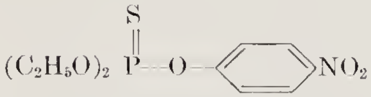
Triphosphoric acid
pentadimethylamide (XIV)


$$[(CH_3)_2N]_3\overset{\overset{O}{\|}}{P}$$

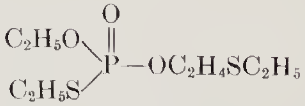
Orthophosphoric acid
trisdimethylamide (XV)



Octamethylpyrophosphoramide *N*-oxide (XVI)



Parathion (XVII)



(XVIII)

Acetylcholine is an important substrate for several of the esterases inhibited by the insecticides discussed in this section. The synthesis of acetylcholine-methyl-$C^{14}$ chloride (IX) by Christenson and Tolbert (64) suggests the possibility of interesting extensions of enzyme, substrate, and inhibitor studies.

*DFP.* Important mode of action concepts for organic phosphates have been discovered using di*iso*propyl fluorophosphate, or DFP (X), a compound which was among the first of the highly toxic, phosphoric acid esters to be discovered. Its action on vertebrates and invertebrates produces symptoms similar to those produced by injecting physostigmine. Michel and Krop (212) tested the possibility of compound formation between cholinesterase and DFP by reacting a cholinesterase preparation from the electric eel, *Electrophorus electricus*, with $P^{32}$-labeled DFP. Radiophosphorus derived from the labeled DFP was bound to protein in amounts directly proportional to the degree of inhibition of the cholinesterase. When another experiment was conducted using $P^{32}$-labeled sodium di*iso*propyl phosphate, no radiophosphorus was bound to the enzyme.

Jandorf and McNamara (151) used $P^{32}$-labeled DFP to study interactions between the compound and proteins of rabbit brain homogenates. The DFP used was prepared by the method of Witten and Miller (317) When labeled DFP was incubated with the homogenates, the initial rate of uptake of $P^{32}$ by the tissue was far higher than the rate of cholinesterase inactivation. Near-maximal binding of the $P^{32}$ was attained at a time when more than 50% of the cholinesterase activity was still unimpaired. A small but significant uptake of $P^{32}$ was obtained when a brain homogenate, previously treated with a 100-fold excess of unlabeled DFP, was incubated with labeled DFP. When the radioactive DFP was subjected to mild alkaline hydrolysis, the resulting mixture added to brain homogenates produced no inhibition of cholinesterase activity and no binding of $P^{32}$ by protein. Jandorf and McNamara (152) also studied the distribution of $P^{32}$ in rabbit tissues following intravenous injection of $P^{32}$-labeled DFP. The $P^{32}$ was retained in relatively large amounts by the kidney, liver, and lungs, while other organs took up only small or insignificant amounts. Of the total amount of DFP-derived $P^{32}$ retained in the above organs, a fraction which reached 100% after 4 hours of survival was bound to tissue proteins. In the plasma, most of the $P^{32}$ originally present was eliminated before regeneration of cholinesterase activity had started. In the erythrocytes, the elimination of $P^{32}$ paralleled the reappearance of cholinesterase, indicating the destruction of cells whose cholinesterase was inactivated by DFP, and their replacement with new cells containing active esterase.

Saunders and Worthy have published preliminary (253) and more detailed (254) accounts of the preparation of $P^{32}$-labeled DFP. It is claimed that this method has advantages over that of Witten and Miller (317).

Jansen *et al.* (153) studied the mode of inhibition of chymotrypsin by using $P^{32}$-labeled DFP. The phosphorus of DFP was introduced into crystalline chymotrypsin by the inhibition reaction of DFP on chymotrypsin. Chymotrypsinogen under the same conditions did not react with DFP. Hence the conversion of the zymogen to the active enzyme liberated not only the groups responsible for activity but also those with which DFP reacts, the two possibly being the same.

Hart *et al.* (129) developed a technique for studying the skin penetration of radioactive anticholinesterases by applying $P^{32}$-labeled DFP to the distal portion of a rabbit ear and placing a Geiger-Müller tube over the proximal portions of the marginal vein.

*HETP and TEPP.* Brauer (47) has found that in the inactivation of human plasma cholinesterase with $P^{32}$-labeled HETP, hexaethyl tetraphosphate (a mixture of ethyl polyphosphates usually containing 12 to 20% of TEPP), no stable combination between the esterase and a phosphorus-containing moiety of HETP took place. He suggested that the mechanism of HETP inhibition involved an exchange reaction between an active hydrogen of the protein and an alkyl group of the inhibitor. Since TEPP (XI), the active constituent of HETP, was found by Jansen *et al.* (153) to be without effect on chymotrypsin, it is possible that some differences between the mechanisms of DFP and TEPP inhibition may exist. Brauer and Pessotti (48,49) used $C^{14}$-labeled HETP to investigate whether the enzyme inhibition involved fixation of any part of the inhibitor molecule upon the inhibited enzyme.

Roan *et al.* (244) and Fernando *et al.* (90) employed several $P^{32}$-labeled organic phosphorus compounds, including TEPP, parathion (XVII), and para-oxon, to study their penetration, distribution, and metabolism in the American cockroach, *Periplaneta americana.* The crop portion of the fore-gut acquired the highest concentrations of radioactivity following either oral or topical administration.

Robinson (248) has reported the preparation of $P^{32}$-labeled TEPP.

*Dimefox.* The absorption of $P^{32}$-labeled dimefox, bis(dimethylamino) fluorophosphine oxide (XII), by the roots of broad bean plants in culture solutions has been studied by David (78). A measurable amount of dimefox absorbed by the roots was given off as vapor by the leaves. The transpired material was collected and shown to be radioactive and systemically insecticidal. Aphids feeding on treated plants contained radioactive material. Dimefox is less lipoid soluble than schradan (XIII) and does not penetrate as readily into the leaves of the broad bean. Since it is also lost by vaporization from the plants, only small amounts were translocated following leaf applications to beans, cabbages, and hops.

The development of techniques for determining plant residues of dimefox, estimated as phosphate, have been reported by Dupée *et al.* (84). Recovery data were obtained by adding known quantities of $P^{32}$-labeled dimefox at an early stage in the analysis procedure.

*Schradan.* $P^{32}$-labeled schradan, octamethyl pyrophosphoramide (XIII), has been applied to plants by various methods in both the laboratory and field. These applications have provided information about the evaporation, absorption, translocation, and decomposition rate and products of this systemic insecticide in plants. It has been generally concluded that the translocation of schradan in plants is greatest in the direction of the most rapidly growing leaves. Enzymatic activation of schradan to a more toxic compound has been discovered in both plant and animal tissues.

Gardiner and Kilby were apparently the first to prepare and use $P^{32}$-labeled schradan (109,110). They performed pilot experiments with the radioactive chemical to determine its absorption and transport in plants. David (76,77) used radioactive schradan, prepared by the method of Gardiner and Kilby, to study the absorption of this systemic insecticide into plants and its action on aphids. The roots of broad beans readily absorbed schradan from culture solutions. The level of radioactivity in the plant increased as the solution was absorbed and was higher in the roots bathed by the solution containing the radioactive schradan than in the rest of the plant. However, the roots also selectively released the labeled portion of the compound, because the radioactivity of the culture solution increased as more schradan was absorbed. The schradan was absorbed more slowly from soil than from sand. In both cases the concentration was highest in the leaves on the middle part of the stem.

When the cut tap-root technique was used to introduce the insecticide, about 50% of the schradan was decomposed within the plant in 8 days. The concentration of undecomposed schradan in the plant necessary to give a complete kill of the bean aphid was about 50 p.p.m. Dead aphids were found to contain about 15 to 20 p.p.m. of radioactive material, calculated on the assumption that it was undecomposed schradan. The honeydew of aphids feeding on treated plants was also radioactive. Absorption and translocation of radioactive material occurred following application to the leaves of the broad bean, cabbage, hop, pea, and strawberry. In the broad bean, radioactive material could be detected within the leaf a few hours after the schradan had been applied to the leaf surface. In all plants there was evidence that radioactivity was translocated to untreated parts. Translocation appeared to be greatest in the direction of the most rapidly growing leaves. No measurable quantity of radioactivity was transpired by plants taking up the radioactive schradan by the roots.

Hartley and Heath (131) have also synthesized $P^{32}$-labeled schradan and have studied its *in vitro* hydrolysis and decomposition in plants.

Hartley *et al.* (132) used $P^{32}$-labeled schradan in their studies of the composition and analysis of the commercial product. Technical schradan was shown to contain about 40% octamethylpyrophosphoramide and a comparable amount of triphosphoric acid pentadimethylamide (XIV). The latter compound was apparently as good a systemic insecticide as octamethylpyrophosphoramide but was much less toxic to mammals. A separate synthesis of triphosphoric acid pentadimethylamide containing $P^{32}$ was carried out for use in biological and analytical tests. About 17% orthophosphoric acid trisdimethylamide (XV) and minor amounts of perdimethylamides of higher polyphosphoric and cyclic metaphosphoric acids were also found in the commercial material. A method of analysis was developed for technical schradan, based upon the various rates of alkaline hydrolysis and differences in the partition coefficients of the compounds listed above.

Heath *et al.* (138,139) studied the decomposition of technical schradan in plants. In one instance (138), plots of strawberries, sugar beets, hops, and Brussels sprouts were sprayed with a radioactive composition containing approximately equal proportions of octamethylpyrophosphoramide and triphosphoric acid pentadimethylamide. The concentration of both compounds in plants fell at much the same rate due largely to decomposition within the plants. The rate of decomposition varied little among the plant species treated providing all were treated at the same time of the year, but the rate of decomposition slowed as the growing season progressed from May to October. In another study (139), sugar beets and strawberry plants were treated with radioactive octamethylpyrophosphoramide and orthophosphoric acid trisdimethylamide. Both compounds were decomposed at similar rates in the two species of plants. When sugar beet plants treated with octamethylpyrophosphoramide were analyzed 10 to 14 days after treatment, it was shown that the $P^{32}$ in the beet existed only as the unchanged amide and products not extractable from water by chloroform. When sugar beet plants treated with orthophosphoric acid trisdimethylamide were analyzed, it was found that, besides the unchanged compound and nonchloroform extractable compounds, some material less extractable by chloroform than the original compound was produced. From these two studies it was theorized that plant enzymes probably had oxidized both parent compounds.

Schradan has been suggested as a control measure to protect groundnut plants against an aphid (*Craccivora* sp.) which is the vector of an economically important virus which infects these plants in the Transvaal (Union of South Africa). Stein *et al.* (273) used $P^{32}$-labeled schradan in the field, at the same time the nonradioactive compound was being used, to study the movement of the insecticide in the plants. A small plot was sprayed by hand and plant samples were taken immediately after spraying and at intervals of a few days, up to 25 days, after spraying. Roots, nuts, and soil samples were tested for total radioactivity only. The decrease in radioactivity in the parts above ground was due to loss of schradan as a whole. The largest loss was due to evaporation immediately after spraying and a further loss took place by movement into the roots, nuts, and soil. There was no decomposition of schradan until more than 18 days after spraying. The radioactivity in the roots and soil samples increased steadily with time.

Wedding and Metcalf (297) have studied the translocation of $P^{32}$-labeled schradan in the bush bean, *Phaseolus vulgaris.* Aqueous solutions containing known amounts of radioactive and nonradioactive schradan were added to the soil of pots containing approximately 3- to 4-week old bean plants. The radioactivity *in situ* was determined by placing a Geiger-Müller tube on the leaves of the living plants. Portions of the plants were sampled at intervals after application of the insecticide and the radioactivity of the samples determined. Extracts from the plants were evaluated for anticholinesterase activity and for toxicity to the two-spotted spider mite, *Tetranychus telarius*. The schradan tended to accumulate most rapidly in the stem and leaves of younger tissues. Movement of schradan through the stem occurred at a rate of about 20 cm./hr. after the insecticide had been taken up from the soil. Less than 1% of the schradan added to the soil was recovered from the above-ground portions of the plant after 120 hours of exposure. The $P^{32}$-containing compounds detected in the plant were toxic to the mites. After 8 days of exposure insecticidally inactive breakdown products of schradan were found.

The behavior of $P^{32}$-labeled schradan has been studied in citrus plants by Metcalf and March (209). When schradan was applied in water culture to the roots of lemon seedlings, the insecticide was readily absorbed and transported to the leaves in rapidly increasing amounts over a 46-day period. Only a small fraction of the schradan was metabolized in the plant to alkali-soluble, chloroform-insoluble products, although the leaf juices became appreciably toxic to the citrus red mite, *Metatetranychus citri*, within 12 to 24 hours. The uptake of schradan was not greatly different from that of $H_3PO_4$ similarly applied. Leaf juices from plants containing relatively large amounts of schradan were only slightly effective as inhibitors of fly brain cholinesterase, and the activity seemed largely independent of the total schradan concentration. This indicates that any toxic metabolite formed is rapidly decomposed and does not accumulate, as does schradan. Additional investigations of this observation were made by Pietri-Tonelli and March (233) and are reviewed later in this section. Although the distribution of schradan was surprisingly uniform throughout the various parts of the plant, the leaves accumulated more schradan than the roots or stems on a weight basis. Translocation appeared to be greatest in the direction of the most rapidly growing leaves. The absorption of schradan applied to the bark at the base of orange seedlings was somewhat greater than from water culture solutions. Applications of schradan to lemon leaves were rapidly absorbed. Although applications of schradan to lemon and orange fruits were absorbed more quickly into the peel than similar applications were into leaves, penetration into the pulp was not great.

Kilby (171), in an eloquent discussion of the biochemistry of schradan, has included experimental data obtained with the $P^{32}$-labeled insecticide. In comparison with other organic phosphorus compounds, schradan was almost completely ineffective as an *in vitro* inhibitor of human red cell cholinesterase. When schradan was injected into rabbits, the animals oxidized the insecticide in the liver to a more powerful anticholinesterase, amine oxide derivative, subsequently designated as octamethylpyrophosphoramide *N*-oxide (XVI) (290).

Glynne Jones and Thomas (114), determined that spray applications of $P^{32}$-labeled schradan to mustard and borage plants resulted in contamination of nectar. A series of nectar samples taken over a 4-week period following spraying showed a progressive decrease in total $P^{32}$ content and also in the amount of schradan present in proportion to the decomposition products. The highest amount of schradan in the nectar was 21 p.p.m. Schradan showed no appreciable breakdown in contact with the honey stomach of the bee and also *in vitro* with the enzyme invertase. Moreover the insecticide was apparently stable in contact with honey over a period of 2.5 months. It was concluded that schradan might appear in an unchanged form in the honey obtained from the nectar of plants which had been sprayed less than 4 weeks previously.

As an aid to their studies of the mode of action of demeton (XIX, XXIII) and schradan, March *et al.* (203) developed two paper chromatographic techniques for the separation and identification of the components of technical demeton and related compounds and a third for technical schradan and related phosphoramide esters. Curves of radioactivity versus $R_f$ values and autoradiograms of paper chromatograms are included for $S^{35}$-labeled technical demeton and $P^{32}$-labeled schradan.

Pietri-Tonelli and March (233) evaluated the toxicity of schradan itself, and of schradan after contact with plant and animal tissues, against several insects and the citrus red mite. $P^{32}$-labeled schradan applied to the stem of a potted Black Valentine bean plant was translocated to the leaves and the concentration of schradan and metabolites in the leaves was determined. Schradan was appreciably toxic by direct contact or ingestion to first-instar nymphs of the cowpea aphid, *Aphis medicaginis*, to adults of the citrus red mite and to third- or fourth-instar larvae of the southern house mosquito, *Culex quinquefasciatus*. No real differences could be shown by bioassay between the toxicity of schradan to these species and the toxicity of comparable amounts of schradan after exposure to the activating processes of plant or animal tissues. These experiments indicate that when small amounts of the active metabolite, such as may be produced in plant tissues, are ingested with considerably larger quantities of unchanged schradan, the former amounts are of little or no importance in relation to total toxicity. The unchanged schradan is metabolized in these arthropods to other compounds which are responsible for the toxicity.

$P^{32}$-labeled schradan has been used by Batt, Bennett, and Thomas to study some of the factors associated with the evaporation, absorption, translocation, and breakdown following foliar application of the insecticide to apple stocks, broad and runner beans, chrysanthemums, and *Coleus*. Batt *et al.* (28) describe the experimental techniques and procedures used in their related studies. Bennett and Thomas (33) found that when radioactive schradan was sprayed on leaves, some was absorbed, some evaporated, and the rest remained on the leaf surface for a considerable time as a residue, removable by aqueous leaching. Absorption of the insecticide proceeded through the leaf cuticle in preference to vapor phase entry through the stomata. The insecticide was generally absorbed more readily by young leaves than by older leaves. Thomas and Bennett (281) studied the translocation and breakdown of radioactive schradan by certain plants. Light aided translocation of the insecticide; the main direction of translocation was upwards. In apples translocation took place mainly in the phloem but some did occur in the xylem, particularly upwards. No major difference was observed in the amounts of schradan translocated or absorbed for *Coleus*, beans, and chrysanthemums but in apples the proportion translocated was greater. The decomposition of schradan into nonchloroform extractables varied between the plant species and was far greater in beans than in *Coleus*. In chrysanthemums and apples the breakdown was similar throughout the plant but in beans and possibly *Coleus* the breakdown products in the untreated sections were higher.

A preparation of $P^{32}$-labeled schradan was sprayed on cotton at the rate of 1.0 pound per acre and after 41 days the extent of contamination of leaves, seeds, raw and refined oils, cake, cottonseed meal, and soapstock was evaluated by Metcalf *et al.* (208). Schradan showed a surprising affinity for the oily seeds, and about 8 to 16 p.p.m. were present in the raw oil. In the refined oil only 0.02 p.p.m. of schradan could be detected; most of the schradan was transferred to the soapstock. Ground cottonseed meal and cake contained 70 to 80 p.p.m. of $P^{32}$ calculated as schradan, but the very low $CHCl_3$/1N NaOH partition ratios indicated that this material was completely metabolized to acidic products. A series of autoradiograms of treated bolls also showed very high concentrations of radioactivity in the seeds.

$P^{32}$-labeled schradan was prepared from $P^{32}OCl_3$ by Heath *et al.* (137) to aid in their investigation of extraction techniques for separating the insecticide from natural products in crop extracts. Labeling the insecticide with radioactive phosphorus made it possible to determine the insecticide and any phosphorus compounds derived therefrom during various operations, regardless of the large amounts of natural phosphorus compounds present. A technique involving low pressure, microdistillation of chloroform extracts of schradan residues was developed which should be applicable to the determination of residues of other pest control compounds of volatility similar to or greater than that of schradan.

*Parathion.* Lockau *et al.* (192) labeled parathion, *O,O*-diethyl *O*-*p*-nitrophenyl thiophosphate (XVII), with $P^{32}$ to determine its uptake by insects and its ability to penetrate into plants. Details of the preparation of the radioactive compound are included in a later paper (191). Cuticular applications to insects were made with a water emulsion of the compound and amounts of parathion sufficient to produce mortality were determined with the American roach and *Drosophila melanogaster.* The penetration into apples of a water emulsion of the radioactive compound was also studied. Radioactivity from the parathion and its metabolites was widely distributed within American roaches topically treated with water emulsions of the insecticide. The highest activities were found in the head and gut. In the head, the radioactivity was associated with the brain and subesophageal ganglia; in the case of the gut, the radioactivity was believed to be due to the excretory function of the gut and associated appendages.

Jensen *et al.* (160) have studied the fate of parathion in rabbits treated dermally and intravenously with $S^{35}$-labeled parathion prepared according to the method of Jensen and Pearce (163). There was very little accumulation of parathion or a sulfur-bearing portion of the molecule in the blood, organs, or tissues. The parathion or sulfur-containing degradation products were rapidly excreted in the urine. The excreted moiety appeared to be an inorganic residue of parathion.

Parathion doubly labeled with $P^{32}$ and $S^{35}$, prepared by Hein and McFarland (140), has been fed in capsules to a goat (72). A comparison was made with a similar administration of $P^{32}$-labeled phosphate to another goat. The radioactivity in samples of milk, blood, urine, and feces taken during the 24 hours following administration of the radioactive parathion and inorganic phosphate was determined. Quite different metabolic pathways were followed by the two chemicals. The inorganic phosphorus appeared in the milk in a manner similar to that reported by Kleiber *et al.* (173). The labeled portion of the parathion molecule began to appear in the urine almost immediately after administration. Dahm and Robbins (72) also used this doubly labeled parathion to study its distribution in the American roach. The high concentration of parathion or a labeled portion of the molecule in the fore-gut, following topical application, led to histological and autoradiographic studies with this organ.

$P^{32}$-labeled parathion was synthesized by Murray and Spinks (222) with a yield of 16% based on $PCl_3$. Parathion and *O*-ethyl *O,O*-di-(*p*-nitrophenyl)-thiophosphonate (NIUIF-101) (VII) have been labeled with $P^{32}$ and $S^{35}$ by Ya *et al.* (319).

*Systox® and Demeton.* In the United States the systemic insecticide formulation known as Systox® is a mixture of an emulsifying agent and demeton, the latter consisting of two closely related thiono and thiol isomers. The insecticidally active isomers in demeton are *O,O*-diethyl *O*-(2-ethylmercaptoethyl)-thionophosphate (XIX) and *O,O*-diethyl *S*-(2-ethylmercaptoethyl)-thiolphosphate (XXIII); these isomers occur in a ratio of approximately 2 to 1, respectively. Radioactive demeton isomers have been used to study the volatilization, absorption, translocation, and decomposition rate of products of the compounds in plants and animals. Generally, the systemic behavior of these compounds resembles that of schradan (XIII); however, the mixture of isomers in technical demeton has presented greater difficulties in tracing their more extended metabolic pathways. Basically, metabolism of the demeton isomers in plants and animals appears to involve a succession of enzymatic oxidations resulting in compounds with anticholinesterase properties greater than their parent isomers. The metabolites ultimately are hydrolyzed to nontoxic residues.

The active constituent of Systox® was stated originally to be *O,O*-diethyl *O*-(2-ethylmercaptoethyl)-thionophosphate (XIX). Several factors, including insolubility in water, toxicity to insects and mammals, and knowledge of thermal instability and isomerization of many compounds of the S═P—O—type to O═P—S—(e.g., parathion), suggested that Systox® might contain, in addition to the above compound, either or both of the isomers *O,O*-diethyl *S*-(2-ethylmercaptoethyl)-thiolphosphate (XXIII) and *O,S*-diethyl *O*-(2-ethylmercaptoethyl)-thiolphosphate (XVIII). In fact, Gardner and Heath (111) have described the preparation and purification of $P^{32}$-labeled thiono (XIX) and thiol (XXIII) isomers. Using essentially an isotope dilution technique, the radioactive isomers were mixed with the active ingredient (demeton) isolated from Systox® and subjected to concurrent partition chromatography. It was established that the thiono and thiol isomers were the principal toxic compounds in Systox® and that the former isomerized to the latter upon heating.

Tietz (284) reportedly employed the $P^{32}$-labeled thiono isomer of demeton (synthesized in the laboratories of G. Schrader, Farbenfabriken Bayer, Elberfeld, Germany) to study its absorption through roots, upward movement, and accumulation in the leaves of plants. The penetration and movement of the radioactive insecticide following application to leaves, and the residual effects, including storage and detoxication by plants, were studied also. The insecticide was absorbed freely from solution by the intact root; when absorption occurred from treated soil, the permeability of the root for water increased over a long period, then dropped below the normal value, and finally adjusted to a normal value. After absorption by the root, the insecticide was translocated to shoot organs growing above the ground. The leaves apparently stored the insecticide temporarily, and the insecticide was accumulated to a greater extent in the regions of the leaf periphery than in the center of the leaf. When a solution of the radioactive insecticide was sprayed or brushed onto leaves, a high concentration of the insecticide penetrated into the leaf rather quickly. Once within the leaf, the insecticide did not appear to diffuse widely. Autoradiograms of leaf cross-sections indicated the insecticide was stored temporarily by the living cells, particularly in the epidermis and in the cells of the connecting bundle parenchyma. The phloem was primarily responsible for translocation of the insecticide following treatment of the shoot part growing above ground, although certain quantities also passed into the xylem. Loss of the insecticide from the plant took place chiefly as the result of exudation through the pores of the cuticle. As an investigation of plant metabolic products of the insecticide was not undertaken the conclusions were based largely upon radioactivity measurements

Chatters (63) used $P^{32}$- and $S^{35}$-labeled Systox® for a number of experiments, including seed treatments and volatilization, translocation, vapor pressure, and phytotoxicity studies.

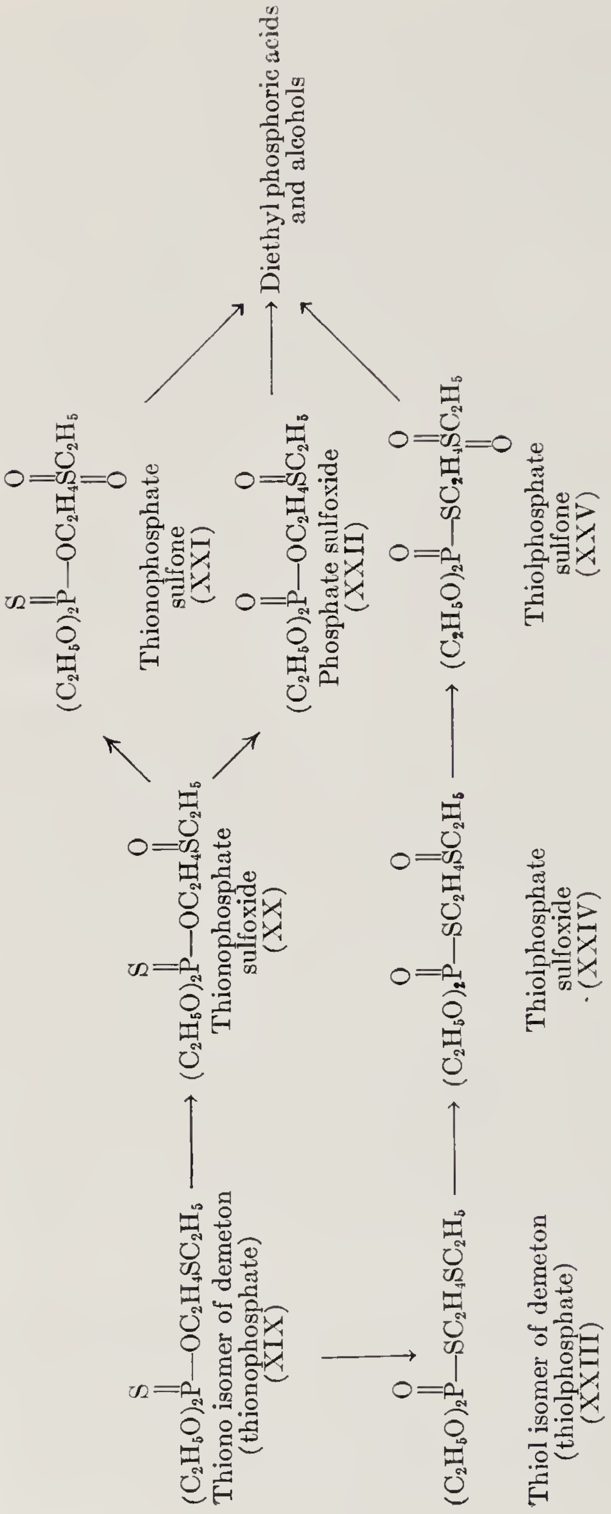
The $P^{32}$-labeled thiol isomer of demeton (XXIII) was used by Stein and Smith (274) to determine the dissipation of residues in tobacco and potatoes at various times after application. The metabolic products of the isomer in plants were not determined, and residues were calculated on the basis of total insecticide applied. A formulation was used having the proportions of 3:7:10, consisting of the thiol and thiono isomers of demeton and emulsifying compound, respectively. Technical demeton was sometimes used to dilute the formulation containing the radioactive thiol isomer. The mixture of the two isomers was used at a concentration of 0.05%. In the case of tobacco, the residues decreased rapidly to a low value at 28 days. Treatment of soil in which tobacco and potatoes were grown indicated that the insecticide or degradation products thereof, were taken up continuously over long periods. Stein (272) also used the $P^{32}$-labeled thiol isomer of demeton to evaluate residues and degradation products of this insecticide in tobacco and potatoes grown in treated soil.

A comprehensive study of the chemistry and metabolism of demeton isomers has been conducted at the Citrus Experiment Station, University of California, Riverside (101,102,103,205,207,210,211). An investigation by Fukuto and Metcalf (101) of the rearrangement of the thiono (XIX) to the thiol (XXIII) isomer of demeton was aided by using $P^{32}$-labeled compounds. This rearrangement was found to occur with heating (130°C.) and shows first-order kinetics. The effect of solvents on the rearrangement was investigated also and since the isomerization was first-order and was enhanced by the more polar solvents, it was predicated that the rearrangement proceeded through an ionic type intermediate. Two possible mechanisms, one involving a sulfonium ion intermediate, were suggested.

Metcalf *et al.* (210) have studied the behavior of thiono and thiol isomers of demeton in bean and citrus plants using radioactive preparations labeled with either $P^{32}$ or $S^{35}$. The isomers were readily absorbed by the roots and stems of lemon seedlings and translocated to the leaves in amounts toxic to the citrus red mite, *Metatetranychus citri,* and greenhouse thrips, *Heliothrips haemorrhoidalis.* The translocated materials concentrated most strongly in the peripheral growing areas of the upper leaves, and the systemic behavior closely resembled that of schradan (XIII). Following topical application of the thiol isomer to stems of bean and lemon plants, radioactivity accumulated in the upper leaves faster than it did following application of the thiono isomer. Both isomers seemed to be rapidly metabolized in bean and lemon leaves. The thiol isomer was several times more toxic to citrus red mites and thrips than was the thiono isomer. The pure thiono isomer was less of an inhibitor of fly brain cholinesterase than was the thiol isomer and the plant metabolites of both isomers. Radioactive vapors could not be isolated from the leaves of bean or lemon plants topically treated on the stem by the labeled isomers.

March *et al.* (205) studied the metabolism of demeton in the white mouse and the American cockroach using both the radioactive thiono and thiol isomers and possible metabolites (102). The thiono and thiol isomers were rapidly metabolized, degraded, and eliminated in both the mouse and the cockroach. The principal route of elimination in the mouse was through the urine, 50 to 70% of an orally administered dose was eliminated within 24 hours, 90% of the eliminated compounds were in the form of water-partitioning, ionic degradation products. The liver was the principal organ of metabolism and degradation. Metabolism of the isomers was somewhat slower in the cockroach. The fore-gut selectively absorbed the compounds and was the principal avenue of elimination. In the roach, tissues such as nerve and muscle apparently played a more active role in the metabolism and degradation of the isomers than the same tissues did in the mouse.

The principal pathway of metabolism for both isomers is the oxidation of the mercapto sulfur of the ethylmercaptoethyl moiety (XIX, XXIII) to the more toxic sulfoxide (XX, XXIV) and sulfone (XXI, XXV) derivatives. In the case of the thiono isomer, a secondary pathway involves the oxidation of the thiono sulfur (XIX) to produce the phosphate (XXIII) and then its sulfoxide (XXIV) and sulfone


$(C_2H_5O)_2P(S)—OC_2H_4SC_2H_5$
Thiono isomer of demeton
(thionophosphate)
(XIX)
$(C_2H_5O)_2P(S)—OC_2H_4S(O)C_2H_5$
Thionophosphate
sulfoxide
(XX)
$(C_2H_5O)_2P(S)—OC_2H_4S(O_2)C_2H_5$
Thionophosphate
sulfone
(XXI)
$(C_2H_5O)_2P(O)—OC_2H_4S(O)C_2H_5$
Phosphate sulfoxide
(XXII)
Diethyl phosphoric acids
and alcohols
$(C_2H_5O)_2P(O)—SC_2H_4SC_2H_5$
Thiol isomer of demeton
(thiolphosphate)
(XXIII)
$(C_2H_5O)_2P(O)—SC_2H_4S(O)C_2H_5$
Thiolphosphate
sulfoxide
(XXIV)
$(C_2H_5O)_2P(O)—SC_2H_4S(O_2)C_2H_5$
Thiolphosphate
sulfone
(XXV)

(XXV) derivatives. Both isomers and their toxic metabolites are degraded by hydrolysis of the P—O— or P—S— bonds to form the alcohol and acid. The routes of metabolism and the toxic metabolites formed are thus the same in plants and animals.

Metcalf *et al.* (211) have considered the nature and significance of demeton residues in plant materials in relation to their previous research with this insecticide (102,205,210). $P^{32}$ radiotracers of the two demeton isomers (101) and the paper chromatography techniques described by March *et al.* (203) were used in these investigations. In orange, apple, and walnut, metabolism of the isomers occurs in the same manner as described previously (102,210). Within 1 or 2 weeks after application of either isomer, almost no trace of the starting material could be found in the plant tissues. Paper-chromatographic techniques indicated the presence of several oxidative products of which the major constituents appeared to be the thiolphosphate sulfoxide (XXIV), the thionophosphate sulfoxide (XX), the phosphate sulfoxide (XXII), and/or the thionophosphate sulfone (XXI), and perhaps the thiolphosphate sulfone (XXV). The action of air and sunlight on surface residues of the isomers appeared to promote their oxidation in the same sequence as found *in vitro* with hydrogen peroxide (102) and in plant tissues.

These oxidative metabolites are subsequently hydrolyzed to nontoxic compounds, including diethyl phosphoric acids and alcohols. Metabolites of the thiol isomer persist in leaf and fruit tissues about twice as long as the thiono isomer metabolites; the respective half-lives of the metabolites in cotton leaves and orange pulp are: thiol isomer, 18 and 30 days; thiono isomer, 8 and 20 days.

If the thiolphosphate sulfoxide (XXIV) and the thiolphosphate sulfone (XXV) are the principal toxic plant metabolites of demeton, residue values obtained by cholinesterase inhibition techniques which employ the thiono and thiol isomers of demeton as standards would appear to be approximately 5 to 10 times too large. This ensues from the observation that the thiolphosphate sulfoxide is about 5 times and the thiolphosphate sulfone about 10 times more active as cholinesterase inhibitors than technical demeton. These investigations with the radioactive isomers of demeton have shown that the average residue values at 2 and 4 weeks after application were substantially below 0.1 p.p.m. and generally in the range of 0.01 to 0.03 p.p.m. for orange juice, total apple peel and pulp, walnut meat, pear, and potato. These amounts are generally of such low magnitude that they could not have been precisely determined by other than tracer techniques.

Fukuto *et al.* (102,103) have unraveled the metabolic fate of the thiono and thiol isomers of demeton in cotton plants. Preparation of $P^{32}$-labeled compounds aided considerably in conducting these investigations. Seven oxidation products of the thiono and thiol isomers of demeton were synthesized and some of their properties compared with the metabolic products obtained after topical applications of the demeton isomers to the base of cotton plants. Utilizing data obtained from paper chromatographic studies, cholinesterase inhibition, systemic activity, and mammalian and insect toxicities of the oxidation and metabolic products, it was discovered that the thiono isomer of demeton (XIX) was converted to the thionophosphate sulfoxide (XX), which was then converted either to the thionophosphate sulfone (XXI), or phosphate sulfoxide (XXII), or to both. The thiol isomer (XXIII) was converted to the thiolphosphate sulfoxide (XXIV) and then possibly to the thiolphosphate sulfone (XXV) (102).

A major metabolic product of the thiol isomer in the cotton plant 4 to 6 days after application was isolated by column chromatography. The infrared spectrum of this metabolite proved conclusively that it was identical to the thiol isomer sulfoxide (XXIV), *O,O*-diethyl *S*-ethyl-2-sulfinylethyl phosphorothiolate. The infrared spectrum of the thiol isomer sulfoxide metabolite isolated from the cotton plant 2 weeks after application of the sulfoxide proved that subsequent oxidation to the thiol isomer sulfone (XXV), *O,O*-diethyl *S*-ethyl-2-sulfonylethyl phosphorothiolate, occurs at a somewhat slower rate than the conversion of the thiol isomer to the sulfoxide (103).

Metcalf *et al.* (207) have compared the systemic behavior of the dimethyl sulfonium salt of the thiol isomer of demeton, designated as the thiol isomer methyl sulfate (XXVI), and the thiol isomer sulfoxide (XXIV), designated as *O,O*-diethyl *S*-2-ethylsulfinylethyl phosphorothiolate, with the thiol and thiono isomers of demeton using cotton plants, $P^{32}$-labeled compounds, and paper chromatography techniques. Following topical applications to the stems of young cotton plants, the accumulation of radioactive materials in the leaves was most rapid with the thiol isomer (XXIII) up to 14 days after which time the thiol isomer sulfoxide treatment gave higher values. The accumulation of radioactivity from the dimethyl sulfonium salt of the thiol isomer (XXVI) was much slower than accumulations from the thiol isomer and its sulfoxide derivative, indicating a lower degree of penetration of the polar sulfonium compound through the plant cuticle. The rates of metabolism and decomposition of the thiol isomer and its sulfoxide derivative were approximately the same and small amounts of thiol isomer sulfone (XXV) were formed from both treatments.

The translocation of Systox® in the cotton plant has been studied also by Ahmed *et al.* (7). Apparently using the mixed isomers of demeton labeled with $S^{35}$, the insecticide was introduced via cut petioles of the plants and radioassays were made of leaves, blossoms, bolls, and seeds. The cotton aphid, *Aphis gossypii,* was used for biological assays of the insecticide and its metabolites. Translocation of the insecticide occurred only in the xylem and was rapid in an upward direction and slightly slower downward. This behavior of the demeton isomers in cotton plants was in contrast with that of schradan and its decomposition products which have been reported (281) to move mainly in the phloem tissue following leaf application to apples. Cotton seed from fruit treated at flowering showed measurable amounts of radioactivity 35 days after treatment although some decrease had occurred during this period of time. Ahmed *et al.* (6) also used labeled Systox® to determine its effect on several species of insects which were predacious on the cotton aphid.

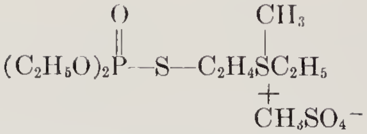
The stability, absorption, and translocation of the $P^{32}$-labeled thiol isomer of demeton were determined by Thomas *et al.* (282) following applications to the leaves of broad beans, *Vicia faba,* a variety of variegated *Coleus,* and apple stocks, and to the roots of broad beans. These workers had reported previously that the insecticidal action of demeton was due very largely to the thiol isomer content. This isomer was unstable after application to plants and degraded to two toxic derivatives referred to as $D_1$ and $D_2$ which further degraded into more hydrophilic compounds designated as $D_3$. The insecticidal action was due mainly to the presence of $D_1$ and $D_2$. Aqueous emulsions of the radioactive insecticide were brushed or sprayed onto leaves. Application to soil was effected by pouring an emulsion evenly over the surface. The pure thiol isomer was chemically stable in daylight and in darkness, but aqueous emulsions were slowly decomposed into more hydrophilic products. This hydrolytic decomposition occurred both in light and dark and the authors emphasized the need for using freshly prepared emulsions when working with this insecticide. In contact with soils the breakdown rate was considerably accelerated; this might have been due to soil organisms as breakdown rates in sand were only slightly higher than in the emulsion. Procedures described by Thomas and Glynne Jones (283) were used to estimate the amount of degradation products; the chemical nature of these products was not determined.

Following application of the thiol isomer to leaves, the concurrent processes of evaporation, breakdown into toxic, nonvolatile compounds, and absorption effectively removed the insecticide from the leaf surface within a few hours. The evaporation resulted in a fumigant action on *Aphis fabae.* Both the thiol isomer and its degradation products were absorbed, the former degrading rapidly within the leaves. Translocation from treated leaves was never sufficient to kill aphids feeding on the untreated foliage. In no instance following leaf application could any unchanged thiol isomer be found elsewhere in the plant. There was a higher proportion of primary toxic degradation products, $D_1$ and $D_2$, in the treated than in the untreated leaves.

Aphids feeding on the shoot tips were being killed 2 days after root application of the thiol isomer to beans in soil or sand. The unchanged isomer was translocated following root application. The isomer and its toxic derivatives appeared to move more freely in xylem than in phloem tissue; movement from xylem to phloem probably occurred, but subsequent transport within the latter tissue was limited. In this connection, when schradan (114) and the thiol isomer of demeton (283) were applied to leaves of borage and mustard plants about to flower, far less thiol isomer and its metabolites appeared in the nectar than was the case with schradan. Assuming that the nectar of these plants was largely secreted by the phloem, the limited movement of the thiol isomer and its metabolites in the phloem seems to be established. There was also evidence that the movement taking place in phloem was mainly one of secondary, more hydrophilic degradation products ($D_3$ above).

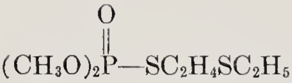
Thomas and Glynne Jones (283) have employed the $P^{32}$-labeled thiol isomer of demeton (demeton-S) to determine the rate of translocation and breakdown of the isomer within plants and if this isomer or its derivatives appear in the nectar of treated plants. A water emulsion of the insecticide was sprayed onto white mustard, *Brassica* *alba*, borage, *Borago officinalis*, and field beans, *Vicia fabae*, when the first flowers opened. Two days elapsed between the last spraying and the first nectar collection and all opened flowers were removed during this period to ensure that no direct contamination of the nectaries occurred. Leaf and bean seed samples were also taken. Estimation of degradation products in the plant was made on samples of diluted nectar and the filtrate from a macerate of plant tissue sample which had been partitioned with various solvents to separate the metabolic products $D_1$ and $D_2$, and $D_3$ described above. The thiol isomer was readily absorbed by the plant tissues and quickly broken down into compounds which appeared elsewhere in the plant, including the nectar. These experiments confirmed Hartley's report (130) of two initial breakdown products. Radioassays of nectar samples from flowers which opened a few days after spraying indicated the absence of the thiol isomer and the presence of only small amounts of the toxic products $D_1$ and $D_2$. Further degradation occurred rapidly. The highest value for total radioactivity found in the nectar corresponded to 2.7 p.p.m. expressed as thiol isomer. When compared with the results obtained in a parallel experiment with schradan, it appeared that the nectar was very slightly contaminated by applications of the thiol isomer of demeton. Radioassay of treated leaves and new growth after spraying confirmed that the thiol isomer was converted rapidly into two primary degradation products, $D_1$ and $D_2$. Further breakdown was more rapid in new growth and appreciable quantities of the two primary degradation products were retained by the treated leaves.

*Methylisosystox*®. The metabolism of Methylisosystox®, *O,O*-dimethyl *S*-(2-ethylmercaptoethyl)-thiolphosphate (XXVII), has been studied by Mühlmann and Tietz (221) in various plants and the white mouse using $P^{32}$-labeled compounds. Two toxic compounds, the sulfoxide (XXVIII) and sulfone (XXIX) isomers of Methylisosystox®, were identified in plants treated with the insecticide. These isomers were reported to be identical with Heath's derivatives $D_1$ and $D_2$. Both Methylisosystox® and its toxic oxidation products in plants were hydrolyzed to nontoxic compounds, dimethyl phosphoric and orthophosphoric acids. The phosphoric acid formed in this manner was used largely by the plant for the synthesis of phosphatides, especially lecithin. The metabolism of this insecticide in plants was strongly correlated with such weather conditions as temperature and light.



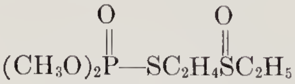
$$(C_2H_5O)_2\overset{\overset{O}{\|}}{P}—S—C_2H_4\underset{\underset{CH_3SO_4^-}{+}}{\overset{\overset{CH_3}{|}}{S}}C_2H_5$$

Dimethyl sulfonium salt of the thiol isomer of demeton (XXVI)



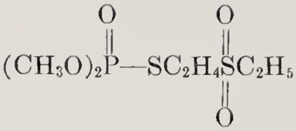
$$(CH_3O)_2\overset{\overset{O}{\|}}{P}—SC_2H_4SC_2H_5$$

Methylisosystox® (XXVII)



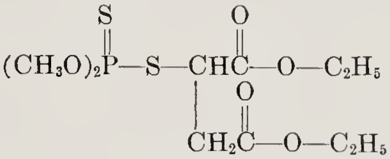
$$(CH_3O)_2\overset{\overset{O}{\|}}{P}—SC_2H_4\overset{\overset{O}{\|}}{S}C_2H_5$$

Methylisosystox® sulfoxide (XXVIII)



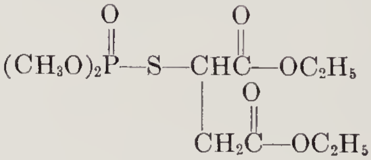
$$(CH_3O)_2\overset{\overset{O}{\|}}{P}—SC_2H_4\underset{\underset{O}{\|}}{\overset{\overset{O}{\|}}{S}}C_2H_5$$

Methylisosystox® sulfone (XXIX)



$$(CH_3O)_2\overset{\overset{S}{\|}}{P}—S—\underset{\underset{CH_2\overset{\overset{O}{\|}}{C}—O—C_2H_5}{|}}{CH}\overset{\overset{O}{\|}}{C}—O—C_2H_5$$

Malathion (XXX)



$$(CH_3O)_2\overset{\overset{O}{\|}}{P}—S—\underset{\underset{CH_2\overset{\overset{O}{\|}}{C}—OC_2H_5}{|}}{CH}\overset{\overset{O}{\|}}{C}—OC_2H_5$$

Thiolphosphate analogue of malathion (XXXI)

*Malathion.* March *et al.* (202) have determined the fate of $P^{32}$-labeled malathion, *O,O*-dimethyl-*S*-(1,2-dicarbethoxyethyl) dithiophosphate (XXX), in the meat and eggs of laying hens, the white mouse, and the American cockroach. Hens were treated by feeding 100 p.p.m. malathion in the mash, by spraying with a 0.5% malathion water emulsion, and by intraperitoneal injection. Residues were determined in the droppings, eggs, and various tissues. Sixty per cent of the radioactive malathion consumed in the feed was eliminated in the droppings in 2 to 4 days and 75% in 5 to 6 days. About 98% of the radioactive compounds excreted was in the form of water-soluble metabolites and degradation products. The maximum total residues found in any of the tissues or eggs examined were less than 3% of the concentration being fed and these residues decreased after the hens were removed from the radioactive feed.

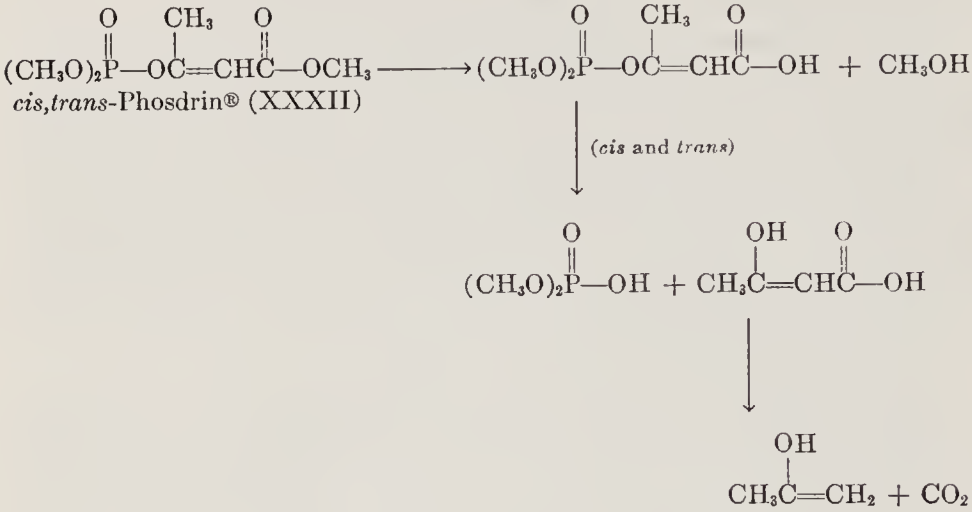
In the case of the sprayed hens, the largest concentration of radioactivity was excreted in the droppings in the first 24 hours after treatment. The maximum concentration in the droppings of the sprayed hens was only about 0.5 that in the droppings of the fed hens. Less than 12% of the applied dose was absorbed and eliminated in 32 days, suggesting that malathion was poorly absorbed through the skin and unavailable for absorption from the feathers. About 90%

of the compounds excreted was in the form of water-soluble metabolites and degradation products. The residues in tissues and eggs were approximately 0.1 of those found in the fed hens.

When a massive dose was injected intraperitoneally into a hen, more than 50% was eliminated in 3 hours, and nearly the entire dose in 24 hours. During the period of greatest elimination, nearly twice as much radioactivity was eliminated in the form of unchanged malathion and chloroform-soluble metabolites as was eliminated in the form of water-soluble metabolites and degradation products. During later periods, 90% or more was excreted as water-soluble metabolites and degradation products. No gross toxic effects were noted in either the fed or the sprayed hens.

The metabolism of malathion in the laying hen and white mouse is complex, probably involving progressive hydrolysis of the ethyl ester moieties to produce more ionic and water-soluble compounds which apparently exhibit a low order of toxicity. It probably also involves the oxidation of the dithio-sulfur moiety to produce a series of thiolphosphates. One of these, *O,O*-dimethyl *S*-(1,2-dicarbethoxyethyl) thiophosphate (XXXI), may be the principal metabolite which shows marked anticholinesterase activity and toxicity. The metabolism of malathion in the American cockroach is apparently less extensive and complex than it is in warm-blooded animals. The less effective metabolism in the insect may explain the great differential in toxicity for malathion between insects and warm-blooded animals.

The fate of $P^{32}$-labeled malathion has been studied in two Jersey heifer calves following the application of two sprays of 0.5% malathion as a water emulsion within a 2-week interval (204). The malathion was rapidly absorbed, metabolized, and 96 to 99% eliminated in the urine principally as water-soluble metabolites and degradation products. The calves were sacrificed 1 and 2 weeks after the second spray application and residues in various cuts of meat and tissues were determined radiometrically. Residues in all tissues, except hide, were in the form of water-soluble metabolites and degradation products. Two weeks after the second spray application only 2.7% remained as unchanged malathion and chloroform soluble metabolites in the hide. Total residues in the meat cuts were low, ranging from 0.05 to 0.15 p.p.m. Higher residues were found in the thymus, thyroid, pancreas, liver, and bone (ranging from 0.2 to 2 p.p.m.). The highest residues were found in the hide, ranging from 3 to 18 p.p.m. Chemical analyses of three cuts of meat showed no detectable malathion (i.e., less than 0.2 p.p.m.) was present.



$$(CH_3O)_2\overset{O}{\overset{\|}{P}}-O\overset{CH_3}{\overset{|}{C}}=CH\overset{O}{\overset{\|}{C}}-OCH_3 \longrightarrow (CH_3O)_2\overset{O}{\overset{\|}{P}}-O\overset{CH_3}{\overset{|}{C}}=CH\overset{O}{\overset{\|}{C}}-OH + CH_3OH$$

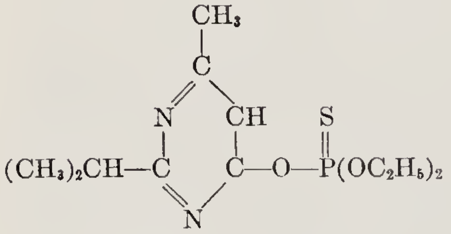
cis,trans-Phosdrin® (XXXII)

$$\big\downarrow \text{(cis and trans)}$$

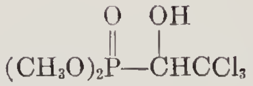
$$(CH_3O)_2\overset{O}{\overset{\|}{P}}-OH + CH_3\overset{OH}{\overset{|}{C}}=CH\overset{O}{\overset{\|}{C}}-OH$$

$$\big\downarrow$$

$$CH_3\overset{OH}{\overset{|}{C}}=CH_2 + CO_2$$



Diazinon (XXXIII)



$$(CH_3O)_2\overset{O}{\overset{\|}{P}}-\overset{OH}{\overset{|}{C}}HCCl_3$$

Dipterex® or Bayer L 13/59 (XXXIV)

*Phosdrin® (OS-2046).* The short-lived residues of Phosdrin®, an insecticidal product containing not less than 60% by weight of the α- or *cis*-isomer of 2-carbomethoxy-1-methyl vinyl dimethyl phosphate (XXXII) have been studied by Casida *et al.* (62) using the $P^{32}$-labeled compound. Phosdrin® consists of about two-thirds *cis*- and one-third *trans*-isomers. The *cis*-isomer is about 100 times more toxic than the *trans*- to insects and mammals. Despite the greater residual persistence of the *trans*-isomer within the plant, its residual hazard was negligible compared to the less stable but more toxic *cis* material. The initial enzymatic attack on both isomers within the plant appeared to be on the carboxylic ester group, followed by hydrolysis of the vinyl phosphate bond according to the scheme shown. The rate of residual loss of Phosdrin® from plants based on radiotracer and anticholinesterase assays, was similar to that based on aphid bioassays. Phosdrin® acts as a systemic insecticide and metabolism within the plant is not required to activate this chemical. The hydrolytic instability of the *cis*- and *trans*-compounds makes a conversion of isomers within the plant appear unlikely. Foliage application to vegetable crops in the field resulted in a 90% residual loss in less than 2 days and over 99% loss in 4 days based on anticholinesterase determinations. The toxic residues of Phosdrin® in crop plants treated at dosage levels used for insect control were essentially dissipated within 2 days following application.

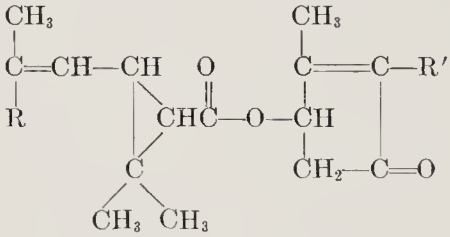
*Diazinon.* The metabolism and excretion of $P^{32}$-labeled Diazinon, *O*,*O*-diethyl *O*-(2-isopropyl-4-methyl-pyrimidyl (6)) thionophosphate (XXXIII), in a lactating Hereford cow have been studied by Robbins *et al.* (246) following oral administration at the rate of 20 mg./kg. The highest concentration of radioactivity in the blood was attained between the 6th and 12th hours after treatment and less than 10% of the radioactivity present through a 36-hour post-administration period appeared to be Diazinon. The remainder of the radioactivity in the blood was due to unknown metabolites and diethyl thiophosphoric acid and/or diethyl phosphoric acid. From 32 to 71% of the radioactivity secreted in the milk from 6 through 24 hours after administration of the radioactive compound appeared to be Diazinon. The maximum concentration of radioactivity occurred in the 18-hour sample. The remainder of the radioactivity appeared to be diethyl thiophosphoric acid and/or diethyl phosphoric acid. The undetermined metabolites found in the blood were not detected in the milk. Only 0.15% of the radioactivity of the administered dose was detected in the milk at the end of 168 hours after treatment.

*Dipterex® (Bayer L 13/59).* The effectiveness against the northern cattle grub, *Hypoderma bovis*, of Dipterex®, *O*,*O*-dimethyl-2,2,2-trichloro-1-hydroxyethylphosphonate (XXXIV) prompted Robbins *et al.* (247) to study the metabolism of the $P^{32}$-labeled compound in a Hereford in order to gain a better understanding of the interrelationships that exist between the host, parasite, and toxicant. The insecticide was administered orally at the rate of 25 mg./kg. and was rapidly metabolized by the cow and eliminated in the urine. About 76% of the radioactivity in the urine was due to an unidentified metabolite, about 17% of the radioactivity in the urine appeared to be dimethyl hydrogen phosphate and/or dimethyl hydrogen phosphite. The major metabolic pathway of this insecticide in the cow apparently does not involve cleavage of the P—C bond in the original molecule.

No dimethyl 2,2-dichlorovinyl phosphate (DDVP) was detected in the blood, milk, or urine. Less than 3% of the Dipterex® administered was accounted for in the fecal samples collected.

### D. PYRETHROIDS AND PIPERONYL BUTOXIDE

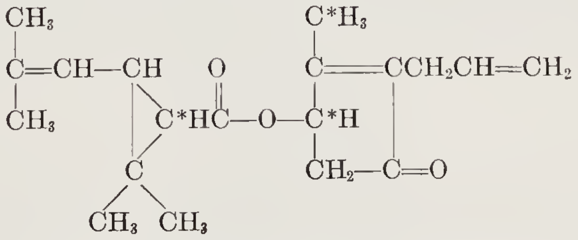
*Pyrethroids.* The flowers of *Chrysanthemum cinerariaefolium* and related species are a rich source of pyrethroid insecticides. The pyrethroids are the most important group of plant-derived insecticides and consist mainly of a mixture of 4 esters and their stereoisomers known as pyrethrins and cinerins (XXXV). For economic reasons, the toxicity of the natural pyrethroids to insects is generally enhanced by the simultaneous application of suitable synergists, particularly certain compounds containing the methylenedioxyphenyl group. These synergists are relatively noninsecticidal.



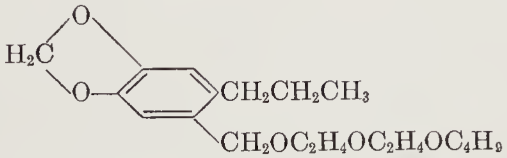
| *Esters* | | *Structures* |
|---|---|---|
| Chrysanthemic esters | | |
| Pyrethrin I | R= —$CH_3$ | R'= —$CH_2CH{=}CHCH{=}CH_2$ |
| Cinerin I | R= —$CH_3$ | R'= —$CH_2CH{=}CHCH_3$ |
| Pyrethric esters | | |
| Pyrethrin II | R= —$\overset{\overset{O}{\|}}{C}$—O—$CH_3$ | R'= —$CH_2CH{=}CHCH{=}CH_2$ |
| Cinerin II | R= —$\overset{\overset{O}{\|}}{C}$—O—$CH_3$ | R'= —$CH_2CH{=}CHCH_3$ |

Principal pyrethroids extractable from
*Chrysanthemum cinerariaefolium* (311) (XXXV)

Pellegrini *et al.* (230) prepared $C^{14}$-labeled pyrethrins and cinerins by growing *C. cinerariaefolium* plants in the presence of $C^{14}O_2$. Earle (85) used these compounds to investigate the synergistic action of piperonyl butoxide. The synergist reduced the detoxication rate of the pyrethroids in several species of insects. Zeid *et al.* (323) employed these radioactive pyrethrins and cinerins to study their tissue distribution and metabolism in American cockroaches. The fore-gut contained the greatest amount of radioactivity per unit of tissue weight after administering lethal, mesothoracic intraspiracular doses of the insecticides. The pyrethrins and cinerins and their hydrolysis products were widely distributed in the insect's body. Winteringham *et al.* (311) have studied the metabolism of $C^{14}$-labeled pyrethroids and allethrin by the housefly, *Musca domestica.* A sample of biosynthesized pyrethoids (230) which was resolved by paper chromatographic analysis (304) contained 41% chrysanthemic esters, 26% pyrethric esters, and 33% unidentified impurities. The $C^{14}$-labeled allethrin was prepared from 1,3-$C^{14}$ acetone (XXXVI). Allethrin, the natural mixture of pyrethroids, pyrethrin I or cinerin I (the latter two chrysanthemic esters (XXXV) were separated chromatographically) were applied topically or injected into groups of houseflies. Comparable fractions of all the pyrethroids were absorbed and metabolized to relatively noninsecticidal substances within 24 hours. When the synergist piperonyl cyclonene was applied simultaneously with the insecticides, their absorption was retarded (presumably by dilution of the pyrethroid on the insect integument) and their metabolisms were all substantially inhibited, but least effectively in the case of allethrin. The synergist seemed to interfere with the natural detoxication mechanism of the fly.



Allethrin. Asterisks indicate introduction of $C^{14}$ in the chrysanthemic acid portion of *dl-cis, trans*-allethrin prepared by Acree *et al.* (4) and in the allethrolone portion of *dl-trans*-allethrin prepared by Winteringham *et al.* (311) (XXXVI)



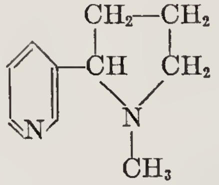
Piperonyl butoxide (XXXVII)

Acree *et al.* (4) prepared *dl-cis,trans*-$C^{14}$-allethrin, the *dl*-allethrolone ester of *dl-cis,trans*-2-$C^{14}$-chrysanthemic acid (XXXVI). The radioactive chrysanthemic acid was prepared from 2-$C^{14}$-glycine. The identification of five contaminants including small quantities of both *dl-cis* and *dl-trans*-2-$C^{14}$-chrysanthemic acids and dimerized allethrolone in this radioactive allethrin was reported by Acree and Babers (2,3). The latter three compounds apparently resulted from traces of allethrin that were hydrolyzed by the ammoniacal solvent during chromatography.

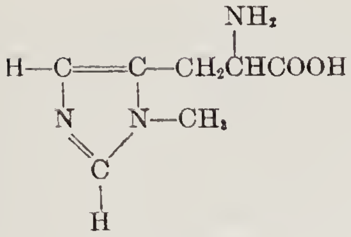
*Piperonyl Butoxide.* Schmidt and Dahm (255) prepared $C^{14}$-labeled piperonyl butoxide, $\alpha$-[2-(2-*n*-butoxyethoxy)-ethoxy]-4,5-methylenedioxy-2-propyltoluene-$\alpha$-$C^{14}$ (XXXVII). About 88% of the piperonyl butoxide applied to Madeira roaches, *Leucophaea maderae,* was absorbed in 3 days; about 50% of the radioactivity was recovered from the feces in 7 days. Paper chromatographic analyses of fecal extracts indicated the presence of less than 50% piperonyl butoxide, the remainder consisted of unidentified, water-soluble metabolites.

### E. NICOTINE

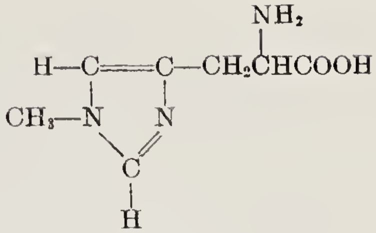
Ganz *et al.* (106,107) have studied the excretion and tissue distribution in several animals of $C^{14}$-labeled nicotine, *l*-1-methyl-2-(3′-pyridyl)-pyrrolidine (XXXVIII), prepared by growing *Nicotiana rustica* plants in the presence of $C^{14}O_2$. In the mouse, the main excretion route was via the urine. No radioactivity was found in the expired air of the mouse. In the rat, the urinary excretion of radioactive nicotine began almost immediately after injection and was practically complete after 16 hours. Brown and Byerrum (52) administered $C^{14}$-methyl-labeled methionine to *N. rustica* plants and established that the methyl carbon of methionine can act as a precursor of the methyl carbon of nicotine. A lesser incorporation of formate carbon into the methyl group of nicotine was observed, suggesting that formate is probably employed by the plant in the synthesis of labile methyl groups, which then undergo transmethyla-



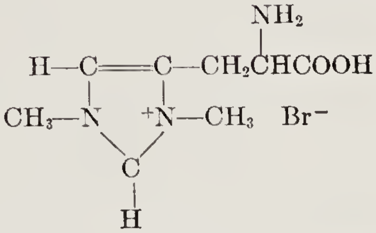
Nicotine (XXXVIII)



1-*N*-methylhistidine
(XXXIX)



3-*N*-methylhistidine
(XL)



1,3-*N*-dimethylhistidine bromide
(XLI)

tion to nicotine. For reasons of structural analogy and biochemical ubiquity, nicotinic acid has been regarded as a possible precursor of the pyridine moiety of the *Nicotiana* alkaloids, such as nicotine. The primary site for the synthesis of nicotine is the root of the plant. However, Dawson *et al.* (80) found that carboxyl-$C^{14}$-labeled nicotine acid and ethyl nicotinate failed to yield isotopically labeled nicotine when supplied to root cultures of *N. tabacum*, var. Turkish.

## F. FUMIGANTS

*Methyl Bromide and Iodide.* The nature and nutritional significance of methyl bromide residues in fumigated wheat were determined at the Pest Infestation Laboratory in England, by Bridges (51), Winteringham (303,306) and Winteringham *et al.* (312). In whole wheat flour, exposed to $C^{14}H_3Br$, the methyl bromide absorbed undergoes decomposition with the formation of inorganic bromide and a series of methylated derivatives. The production of free methanol as a result of hydrolysis was about 10% or less. The rate of methyl bromide decomposition was greater at lower moisture levels, indicating that simple hydrolysis was not an important factor in decomposition. The gluten or protein fraction of the flour contained about 80% of the absorbed and decomposed methyl bromide. Methylated decomposition products were due almost entirely to *N*-methyl (50%), dimethyl sulfonium (30%), and methoxyl (20%)

derivatives. The principal reaction between $C^{14}H_3Br$ and the *N*-containing groups of wheat protein was highly specific, being mainly one of methylation of the imidazole ring of histidine. Three compounds, the 1- and 3-*N*-methylhistidines (XXXIX, XL) and 1,3-*N*-dimethylhistidine bromide (XLI), accounted for about 75% of the total *N*-methylation products. About 10% of the total *N*-methylation occurred as ε-*N*-methylated lysine. No appreciable loss of any of the essential amino acids in wheat protein occurred by *N*-methylation when wheat was fumigated under normal conditions. There was no evidence that the principal fumigant decomposition products (including such end products of digestive hydrolysis as methanol, methyl glucoses, *S*-methyl cysteine, methyl methionine sulfonium salts, and *N*-methyl derivatives of histidine and lysine) are toxic or that their formation would be associated with any significant reduction in essential food constituents such as vitamins. Winteringham (302) synthesized $CH_3Br^{82}$ and studied its behavior as a fumigant; Foreman *et al.* (96) synthesized $C^{14}H_3Br$.

Winteringham and Hellyer (315) compared the effects of methyl bromide, ethylene dibromide, and ethylene dichloride on $P^{32}$-labeled intermediates extracted from the thoracic muscles of houseflies. The slow depletion of phosphoglycerate by the first two chemicals suggested a common inhibition of triose phosphate dehydrogenase, and thus they resembled iodoacetate in their action. Depletion of ATP and arginine phosphoric acid by methyl bromide indicated a rapid blocking of the phosphorylation of nucleotide acceptors.

Winteringham *et al.* (313) prepared $I^{131}$-labeled methyl iodide by an exchange reaction with carrier-free $I^{131}$ and used the compound to study its penetration and distribution by autoradiography in larvae of the common bluebottle fly, *Calliphora erythrocephala*. Tolbert (285) synthesized $C^{14}H_3I$.

*Carbon Disulfide.* The distribution and excretion in guinea pigs and mice of $CS_2^{35}$ has been reported by Strittmatter *et al.* (277). Intracardially injected carbon disulfide was retained by guinea pigs in direct proportion to the size of the dose. There was a general distribution of carbon disulfide throughout the body tissues following injection or inhalation of the compound, but more $S^{35}$ was present in the liver than in any other tissue studied. Although nonmetabolized carbon disulfide was excreted rapidly, the body tissues retained large quantities of the $S^{35}$ and gave them up slowly. About 30% of the carbon disulfide retained was excreted in the urine, largely as inorganic sulfates; from 5 to 15% was metabolized and excreted by the feces.

*Carbon Tetrachloride.* McCollister *et al.* (198) used $C^{14}Cl_4$ prepared according to the method described by Beamer (31), to determine the metabolism of carbon tetrachloride by monkeys exposed to low vapor concentrations. Of the tissues examined, the highest concentration of radioactive material was found in the fat; radioactive carbon was found also in the blood carbonate, exhaled carbon dioxide, and urinary urea and carbonate. Most of the radioactivity in the urine appeared to be present in some nonvolatile fraction other than urea, carbonate, or amino acids. The equivalent of at least 51% of the carbon tetrachloride absorbed during an inhalation period was estimated to have been eliminated in the expired air of a monkey within 75 days following the end of the exposure. The remainder was excreted to a large extent in the urine and feces. In other experiments in which monkeys received skin exposures to radioactive carbon tetrachloride vapor, negligible amounts of radioactive material were found in samples of blood and expired air.

*Other Fumigants.* Cox and Warne (69) and Tomisek and Mahler (288) have synthesized ethylene oxide-1,2-$C^{14}$. Of several reported syntheses of $HC^{14}N$ (1,193,197,260) that of McCarter (197) appears to be simplest and least expensive. Belleau and Heard (32) have synthesized $NaC^{14}N$. In these cases the radioactive cyanide radical was prepared for the synthesis of organic compounds. A tedious method for preparation of $S^{35}O_2$ has been reported by Johnson and Huston (164).

### G. MISCELLANEOUS INSECTICIDAL CHEMICALS

*Elemental Sulfur.* Turrell and Chervenak (291,292) mixed $S^{35}$ with elemental sulfur and used the combination to dust lemons and study sulfur metabolism by these citrus fruits. A large proportion of the sulfur applied to the fruit was converted to hydrogen sulfide and sulfate in the fruit peel. Thomas (280) conducted fundamental physiological studies on the role of sulfur, as $S^{35}$, and arsenic, as $As^{76}$, in the economy of a number of crop plants.

*Arsenic.* Campbell and Lukens (60), using $Pb^{212}$ (ThB) as an indicator in acid and basic lead arsenates, found that the acid compound was more soluble than the basic compound in the gut of the silkworm, *Bombyx mori,* and thus provided one explanation why acid lead arsenate is more toxic than the basic compound to many insects. Morrison and Oliver (220) found that injections of arsenic trioxide, containing $As^{76}$, into yellow mealworms, *Tenebrio molitor*, resulted in a rapid distribution of radioactivity throughout the insects. When tomato hornworms, *Protoparce quinquemaculata*, were fed arsenic trioxide they absorbed very little from the intestinal tract. The largest portion either remained in the gut if the larva died or was eliminated if it lived. Norton and Hansberry (223) labeled disodium, basic calcium, and lead arsenates with $As^{74}$ for administration to silkworms.

Ducoff *et al.* (83) have studied arsenic excretion in man, rats, and rabbits using $As^{76}$ prepared by neutron irradiation (Szilard-Chalmers reaction) of cacodylic (dimethylarsinic) acid, $(CH_3)_2AsO(OH)$, according to the method of Straube *et al.* (276). Less than 10% of the arsenic excreted was found in the feces of any of the species employed; rats had by far the slowest rate of excretion. Data are also given for arsenic distribution in various organs in man, the rat, the rabbit, and 2 strains of mice.

Lanz *et al.* (178) studied the metabolism of pentavalent arsenic in laboratory animals using $As^{74}$ as a tracer. Injected doses of arsenic were excreted chiefly by the kidney, 85–90% appeared in the form of arsenate and the other 10–15% appeared as arsenite. The greatest amount of arsenic was recovered from the urine in the first 24 hours. Arsenic in the blood of the rat was almost entirely confined within the erythrocytes and was apparently in stable combination with hemoglobin, mostly associated with the protein globin. The mean life of arsenic in the blood stream of the rat appeared to parallel the mean life of the erythrocytes. There appeared to be very little recombination of radioarsenic with other cells after the original host cell was destroyed. The red cells of the cat concentrated arsenic to as much as 5.6% of the injected dose, whereas these cells in the rabbit, dog, mouse, guinea pig, and chick did not concentrate arsenic to any significant degree. The skeletal muscle contained the largest concentration of arsenic of any tissues from these animals.

Smales and Pate (265,266) have determined by radioactivation amounts of arsenic as small as $10^{-10}$ g. in biological material such as human hair, nails, urine, and blood and the internal organs of a mouse. The normal level of arsenic in human blood or urine can be determined in a 1-drop sample.

*Dinitrophenol.* The effects of 2,4-dinitrophenol (DNP) on the respiration of tissues are accompanied by changes in the acid-soluble phosphorus fractions. Sacks and Sinex (252) used dilutions of $P^{32}$-phosphate to study the effects of graded concentrations of DNP on respiration and phosphorylation in the isolated rat diaphragm. Phosphocreatine was completely hydrolyzed, the concentration of adenosine triphosphate was reduced markedly without regard to whether respiration was stimulated or depressed, and the concentration of hexose monophosphate (HMP) was reduced when the DNP was present in sufficient concentration to depress respiration. The effect of DNP on the turnover of HMP suggested that there were pathways for the formation of HMP in the intact cell which were not sensitive to the depressant effect of DNP.

*Dimethyl Phthalate.* Barker and Christian (26) have synthesized $C^{14}$-carbonyl-labeled dimethyl phthalate.

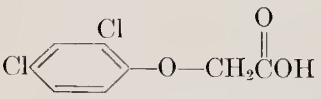
## III. Fungicides

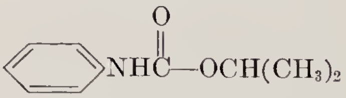
The use of radioactive tracers in studying fungicidal action has been thoroughly reviewed by McCallan and Miller (196), Kemp *et al.* (167), Miller (213), and Miller and McCallan (214). Miller (213) has written: "The effectiveness of agricultural fungicides has been measured for many years in terms of plant disease control or concentration in suspension or solution required to inhibit spore germination or mycelial growth. Such data tell very little about the actual dose taken up by the spores or mycelium or whether the fungitoxicant is taken up by host tissue. The whole problem of perfecting plant protectants would be advanced by a better understanding of what happens to the fungicide after it is applied to the foliage or fruit. It is important to have information on how effective fungicides are on a spore weight basis, whether different species of fungi differ in their ability to take up fungitoxicants and in the lethal doses required, how firmly bound the fungitoxicants are after uptake by the spores and whether different toxicants compete for the same receptor sites, what chemical changes, if any, fungitoxicants undergo after absorption by fungus spores, and whether the toxicants are taken up by host plants and exert any protective action in host tissue. These and similar question have remained essentially unanswered since extensive studies were begun on the development of better fungicides about 1910. The availability of radioactive tracer techniques has made possible investigations which would be extremely difficult or impossible to carry out by straight chemical approaches.

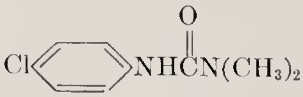
"Approximately 15 labeled fungitoxic compounds or ions have been used in various investigations of fungicidal action. They have been used to determine uptake by fungus spores and to determine the quantities necessary, on a spore weight basis, to prevent germination. Absorption by host plants of labeled fungicides has also been studied. By using more than one toxicant at a time competition for receptor sites in fungus spores has been investigated. Changes undergone by absorbed fungitoxicants have been studied by means of exchange reactions and by the demonstration that $C^{14}O_2$ is formed from some $C^{14}$-labeled fungicides. With the use of $P^{32}$ labeled spores it has been found that some toxicants markedly increase the outward movement of cell contents. Studies with radioactive toxicants and nutrients have shown marked accumulation of the applied material at infected sites of leaves, especially with obligate parasites. This marked accumulation at infected sites suggests that internal therapy should offer promise for the control of these diseases.

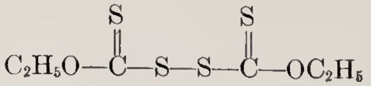
"As labeled fungicides become more available and a greater number of laboratories interested in fungicidal action become equipped to use radioisotopes, the use of tracer techniques in this field will increase greatly. Many of the problems associated with the control of plant diseases are especially susceptible to attack by these techniques."

## IV. Herbicides and Plant Growth Regulators

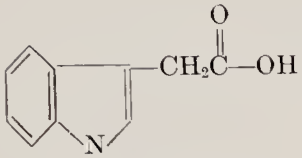

2,4-D (XLII)


IPC (XLIII)

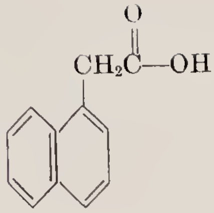

CMU (XLIV)


$C_2H_5O-\overset{\overset{S}{\|}}{C}-S-S-\overset{\overset{S}{\|}}{C}-OC_2H_5$

Bis(ethyl xanthic) disulfide (XLV)



3-Indoleacetic acid
(XLVI)



1-Naphthalene acetic acid
(XLVII)

Phenoxyaliphatic acids, because of their use as plant growth regulators and weed killers, have been the subject of many investigations. With the introduction of 2,4-D, 2,4-dichlorophenoxyacetic acid (XLII), as a derivative widely used for ridding cereals and other crops of weeds, an intensive research program was undertaken to expand the knowledge of this series of compounds. To aid in this effort, several radioactive phenoxyaliphatic acid derivatives have been prepared. There are reports of the preparation of 2,4-dichlorophenoxyacetic acid-1-$C^{14}$ (147,200), and 2,4-dichlorophenoxy-1-$C^{14}$ acetic acid prepared from benzoic acid-1-$C^{14}$ (92). Radioactive 4-chloro-2-iodophenoxyacetic acid (165) and 2,4-dichloro-5-iodophenoxyacetic acid (200,318) labeled with $I^{131}$ have also been prepared. Holley *et al.* (147) applied carboxyl-labeled radioactive 2,4-D to leaves of bean plants and found that the chief movement of radioactivity was downward in the stems. Some $C^{14}$ was lost from the plants to the nutrient solution and as $C^{14}O_2$. The radioactive material in the stems of the treated plants consisted of about one-third 2,4-D; over half of the radioactive material isolated from the stems was not 2,4-D but was water soluble and could not be extracted with ether. Jaworski and Butts (154) have demonstrated that about 60% of the $C^{14}$ from radioactive 2,4-D was in the form of a 2,4-D complex 4 days after treatment of plants. Miller *et al.* (215) compared the volatility of the *n*-butyl, 2-ethylhexyl, and 2-(2-ethoxyethoxy) propyl esters of 2,4-D labeled with $C^{14}$ in the carboxyl position. The *n*-butyl ester was the most volatile, the relative volatility of the 3 esters was approximately 50, 3, and 1, respectively. Mitchell and Linder (216) applied 2,4-dichloro-5-iodophenoxyacetic acid labeled with $I^{131}$, and the morpholine salt of this compound, to bean plants and determined the radioactivity in other parts of the plant 3 days later. The greatest portions of both compounds were found in the first internode, that part of the main stem just below the primary leaves, and the lowest concentrations were found in the roots. Addition of a surface active agent, Tween® 20, increased both absorption and translocation, raising the amount of salt moved to the roots by 71% (an effect of interest in connection with the use of 2,4-D and related compounds in killing deep-rooted perennial weeds).

The herbicide IPC, *O*-isopropyl *N*-phenyl carbamate (XLIII), has been used as a selective grass killer; it has been effective when applied to roots and hence is used for soil applications, usually as a pre-emergence spray. Baldwin *et al.* (23) applied $C^{14}$-labeled IPC (labeled on the secondary carbon of the isopropyl group) to the leaves and roots of corn, *Zea mays*, and oats, *Avena sativa*, and found a descending rate of absorption for the compound through cut leaf surfaces, cut root surfaces, and intact roots. Intact leaf surfaces did not absorb the IPC readily. The absorption through roots was greater in corn plants than in oat plants. These experiments confirmed that IPC is normally absorbed by the roots, not by intact leaves. The authors suggested that soil application or application of IPC immediately after cutting the grass should give better control of weeds. It was not determined whether the radioactivity absorbed and translocated was due to IPC or some metabolic product. Logan and Murray (194) have prepared $C^{14}$-labeled IPC.

The compound 3-(*p*-chlorophenyl)-1,1-dimethyl urea, or CMU (XLIV), has been employed as a pre-emergence herbicide and soil sterilant. It possesses a long residual activity in soil. Fang *et al.* (89) applied carbonyl-$C^{14}$-labeled CMU to the foliage of bean plants, *Phaseolus vulgaris*, and observed that the compound was quickly absorbed and translocated throughout the entire leaf; translocation to other parts of the plant was much slower. By means of paper chromatography it was determined that two major radioactive compounds occurred in leaf extracts; one was CMU and the other was an unidentified CMU complex. Acid hydrolysis apparently split the CMU complex, and the carbonyl-$C^{14}$-CMU decomposed to form *p*-chloroaniline, dimethylamine, and $C^{14}O_2$.

$S^{35}$-containing bis(ethyl xanthic) disulfide (XLV) was used to evaluate the pre- and post-emergence herbicidal action of this compound (177). When an herbicidal formulation containing the radioactive compound was applied as a pre-emergence spray on muck soil, no radioactivity could be detected in any of 9 vegetables grown in the treated soil. Foliar applications of a post-emergence formulation to 16 vegetable crops showed that only in cabbage and cauliflower was translocation apparent. The bis(ethyl xanthic) disulfide acted primarily as a contact herbicide; it was not absorbed by the roots of plants grown in soil treated with a pre-emergence spray and was not translocated by a majority of vegetable plants treated with a post-emergence spray.

Following the discovery that indoleacetic acid was a naturally occurring plant auxin, a great deal of work was done on the physiological and biochemical effects of this compound on plants. This led to the synthesis of many homologs and analogs of indoleacetic acid and the discovery that many of these compounds were powerful plant growth regulators. Both 3-indoleacetic acid-2-$C^{14}$ acid (XLVI) (278) and 1-naphthaleneacetic acid-2-$C^{14}$ acid (XLVII) (5) have been synthesized.

Mitchell and Linder (217) have described methods used in tracing radioactive growth regulating substances in plants.

## V. Tagging Insects, Mites, and Ticks with Radioisotopes

A popular entomological use of radioisotopes has been the tagging of insects and related arthropods (72,156,158,184). To be good for tagging, an isotope should be inexpensive, easy to apply and manipulate, accurately recognizable throughout the life of the tagged organism, and acceptably free from harmful biological effects to both organism and experimenter. Often large numbers of flying insects, such as flies and mosquitoes, have been tagged and released for subsequent capture for determining their migratory habits; cockroaches, plum curculios, wireworms, and ticks have been tagged to permit following their movements under natural conditions. An increased knowledge of the movements of insect pests gained by these methods has helped entomologists plan more efficient pest control programs. Radioisotopes also have been used for the study of the feeding habits of insect and mite vectors of plant diseases to help discover useful relationships between vector, causal organism, and host. A variety of insect tagging experiments have been listed in Table I.

## TABLE I

Tagging Insects, Mites, and Ticks with Radioisotopes

| Insects and related arthropods | Life stages[a] | Radioisotopes and references |
|---|---|---|
| Thysanura | | |
| Firebrat, *Thermobia domestica* | A | $P^{32}$ (190) |
| Orthoptera | | |
| American cockroach, *Periplancta americana* | A | $P^{32}$ (258) |
| German cockroach, *Blattella germanica* | N | $P^{32}$ (190) |
| Grasshopper, *Camnula pellucida* | N | $P^{32}$ (104) |
| Locusts, *Locusta migratoria* and *L. pardalina* | N, A | $P^{32}$ (169,170) |
| Homoptera | | |
| Aphids, *Anuraphis* sp. | N, A | $P^{32}$ (231) |
| Bean, *Aphis fabae* | N, A | $P^{32}$ (36) |
| Cabbage, *Brevicoryne brassicac* | N, A | $P^{32}$ (81) |
| Green peach, *Myzus pcrsicae* | N, A | $P^{32}$ (36,81,179,295) |
| | | $Po^{212}$ (126) |
| Mealybugs, spp. | N | $P^{32}$ (68) |
| Pineapple, *Pseudococcus brevipcs* | N | $C^{14}$ (125) |
| | N | $P^{32}$ (61) |
| Potato psyllid, *Paratrioza cockerelli* | N, A | $P^{32}$ (235) |
| Hemiptera | | |
| Harlequin bug, *Murgantia histrionica* | A | $P^{32}$ (71) |
| Squash bug, *Anasa tristis* | A | $P^{32}$ (71) |
| Tarnished plant bug, *Lygus lineolaris* | A | $P^{32}$ (93,94) |
| Coleoptera | | |
| Boll weevil, *Anthonomus grandis* | A | $Co^{60}$ (19) |
| Coccinellidae | L | $Ta^{182}$, $Ra^{226}$ (24,25) |
| Douglas-fir beetle, *Dendroctonus pseudotsugae* | A | $P^{32}$ (88) |
| Plum curculio, *Conotrachelus nenuphar* | A | $P^{32}$, $Sr^{89}$, $Co^{60}$, $I^{131}$ (241) |
| Rice weevil, *Sitophilus oryza* | A | $P^{32}$ (261) |
| Wireworms, *Melanotus* sp. | L | $Co^{60}$ (99) |
| Prairie grain wireworm, *Ctenicera aeripennis destructor* | L | $Co^{60}$ (14,105) |
| *Agriotes* sp. | A | $Ra^{226}$ (50,287) |
| Yellow mealworm, *Tenebrio molitor* | L | $Na^{22}$ (227) |
| | A | $P^{32}$ (71) |
| | L, A | $P^{32}$ (190) |

TABLE I (*continued*)

| Insects and related arthropods | Life stages$^{a}$ | Radioisotopes and references |
|---|---|---|
| Lepidoptera | | |
| *Arctia caja* and *Panaxia dominula* | L | $S^{35}$ (168) |
| Cutworms, pale western, *Agrotis orthogonia* and Red-backed, *Euxoa ochrogaster* | L | $Co^{60}$ (105) |
| Greater wax moth, *Galleria mellonella* | L | $P^{32}$ (190) |
| Silkworm, *Bombyx mori* | L | $P^{32}$ (320) |
| Hymenoptera | | |
| Honey bee, *Apis mellifera* | A | $C^{14}$ (224) |
| Hornets, *Vespula* spp. and wasp, *Polistes* sp. | L, P, A | $Mn^{52}$, $Mn^{54}$, $Mn^{56}$, $Ba^{140}$ (38,39,40) |
| Diptera (flies) | | |
| Black blowfly, *Phormia regina* | A | $P^{32}$ (188,250,256,322) |
| Black flies, *Simulium* spp. | L | $P^{32}$ (98) |
| *Drosophila gibberosa* | L, A | $I^{131}$ (299,300) |
| *D. melanogaster* | L, P, A | $P^{32}$ (15,16,27,37,149,172) |
| | A | $P^{32}$ (234) |
| *D. repleta* | A | $P^{32}$ (234) |
| | L | $Ba^{140}$, $La^{140}$ (41) |
| *D. virilis* | L, P, A | $P^{32}$ (37) |
| *Eucalliphora lilaea* | A | $P^{32}$ (250) |
| *Fannia pusio* | A | $P^{32}$, $Ca^{45}$, $I^{131}$ (239) |
| Flies spp. | A | $P^{32}$ (267) |
| Green bottle fly, *Phaenicia sericata* | L, A | $P^{32}$ (143) |
| | A | $P^{32}$ (188) |
| | A | $P^{32}$, $Ca^{45}$, $I^{131}$ (239) |
| *Phaenicia* spp. | A | $P^{32}$, $Ca^{45}$, $I^{131}$ (238,239) |
| | A | $P^{32}$ (322) |
| House fly, *Musca domestica* | L, P, A | $P^{32}$ (18) |
| | L, A | $P^{32}$ (143) |
| | A | $P^{32}$ (161,188,238,250,257,259,263,322) |
| | A | $P^{32}$, $Ca^{45}$, $I^{131}$ (239) |
| *Hylemya* spp. | | |
| Cabbage maggot, *H. brassicae* | L, A | $P^{32}$ (225) |
| | A | $P^{32}$ (95) |
| Turnip maggot, *H. floralis* and *H. cana* | L, A | $P^{32}$ (225) |
| Oriental fruit fly, *Dacus dorsalis* | L, A | $P^{32}$ (243) |
| *Sarcophaga sueta* | A | $P^{32}$, $Ca^{45}$, $I^{131}$ (239) |
| Screw-worm, *Callitroga hominivorax* | L, A | $P^{32}$ (240) |
| Secondary screw-worm, *C. macellaria* | A | $P^{32}$, $Ca^{45}$, $I^{131}$ (238,239) |

*Table continued*

TABLE I (*continued*)

| Insects and related arthropods | Life stages[a] | Radioisotopes and references |
|---|---|---|
| Diptera (mosquitoes) | | |
| *Aedes* spp. | L, A | $P^{32}$ (262) |
| *A. aegypti* | L, A | $P^{32}$, $Sr^{89}$ (55)<br>$P^{32}$ (133,134) |
| *A. communis* | A | $P^{32}$ (159,298) |
| *A. excrucians* | L, P, A | $P^{32}$ (155) |
| *A. pullatus* | L, P, A | $P^{32}$ (155) |
| *A. sticticus* | A | $P^{32}$ (321) |
| *A. taeniorhynchus* | L, A | $P^{32}$ (236) |
| *Anopheles* spp. | L, A | Th (11,12,13) |
| *Psorophora confinnis* and *P. discolor* | L, A | $P^{32}$ (237) |
| Mites | | |
| Two-spotted spider mite, *Tetranychus telarius* | E, A | $P^{32}$ (249) |
| Ticks | | |
| Lone star tick, *Amblyomma americanum* | A | $P^{32}$ (174) |

[a] E = egg, N = nymph, L = larva, P = pupa, A = adult.

## VI. Epidemiology and Public Health

It has been written, "In the field of epidemiology of communicable diseases, when significant progress is made, the results are measured in human lives and comfort. One need only recall the scourges of bubonic plague, yellow fever, and epidemic typhus, or the great reductions in malaria, dengue, or enteric diseases to illustrate the importance of controlling arthropods that transmit disease" (156). Radioisotopes have been used to tag arthropods for ecological studies of dispersal and flight range, longevity, hosts and feeding habits, predation, and parasitism. From these applications it has been possible to determine the epidemiological role of arthropods more accurately. Although a few internal parasites of animals have been made radioactive, less attention has been given to tagging ectoparasites such as ticks, fleas, lice, and mites by injecting the vertebrate host animal with radioisotopes. The tabular summary of insects, mites, and ticks in this chapter lists many examples of the use of radioisotopes in public health studies. Additional studies of disease transmission by arthropods, using radioactive pathogens, offer a promising line of investigation; certain nematodes, protozoans, bacteria, and viruses have been made radioactive and are suitable for this purpose. Many advantages of using radioisotopes for studying the epidemiology of communicable diseases have been summarized by Jenkins (156,157).

Specific examples of accomplishment in this field include the following: (*a*) labeling medically important arthropods to determine dispersal range and improve control methods, e.g., American cockroaches did not migrate in a sewerage system (258), the dispersal range of black blowflies (188,256,322), houseflies (188,238,239,257, 259,322), and *Phaenicia* blowflies (188,238,239,322) in rural areas was several miles greater than had been shown by earlier studies, and the movement of several species of medically important and pest mosquitoes has been determined (11,55,159,236,237,262), (*b*) delineating the role of actual and potential vectors of communicable diseases, e.g., the role of houseflies (263) and *Drosophila* (234) in carrying diseases from outdoor privies to houses, (*c*) determining the abundance of natural populations by releasing a known number of radioactive insects in an area, collecting from the natural population and calculating the total population from the ratio of recaptured tagged insects to the total number of collected insects of the same species, and (*d*) studying the metabolic relationships between radioactive internal parasites and their host animals.

## VII. Food Preservation and Pest Control by Radiation Effects

The basic method for preserving foods by heat sterilization has remained the same for the last 150 years despite a continuing search for some heatless method of sterilization. Radiation sterilization appears to have considerable potential in the food industry provided several problems can be solved. These problems deal mostly with the acceptability of irradiated foods from a nutritional and taste standpoint, the economy of the process on a commercial scale, and safety engineering problems. The most useful sources of radiation have been cathode rays produced by a Van de Graaff generator or resonant transformer, x-rays produced by directing cathode rays on a metal target, and $\gamma$-rays from atomic fuel wastes (53,116) (see Fig. 1). Cathode rays have been used in the energy range of 1 to 3 million electron volts (MEV) and are characterized by their rather shallow penetration of substances. X- and $\gamma$-rays are true electromagnetic radiations and have similar physical characteristics including the ability to penetrate substances readily. The $\gamma$-rays are emitted by

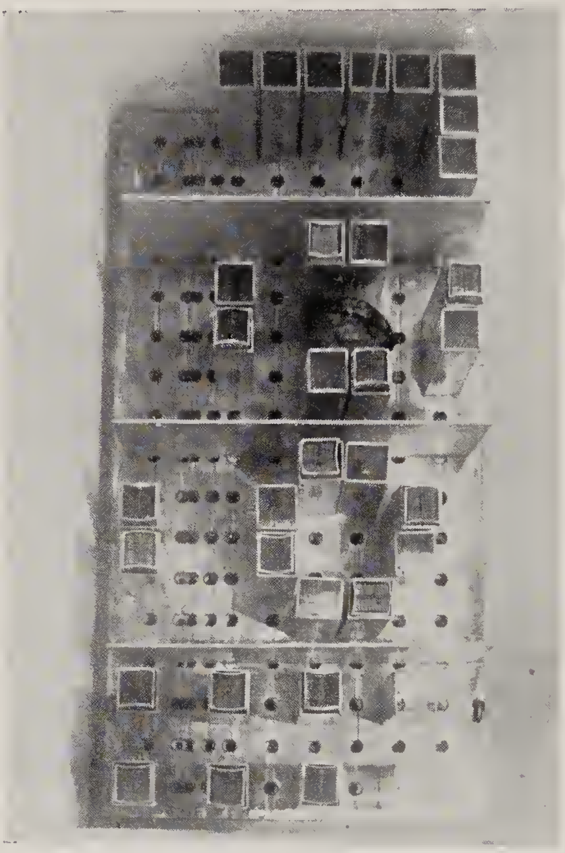


Fig. 1. View from above of the universal-type gamma irradiation grid located in the canal of the Gamma Irradiation Building at the Atomic Energy Commission's National Reactor Testing Station, Idaho Falls, Idaho. The grid consists of an array of spent Materials Testing Reactor fuel assemblies, powerful sources of gamma rays. The active (fuel-containing) portions of these elements are placed in holders that can be plugged into holes in the grid base. Samples of different sizes and shapes can be accommodated by rearrangement of the elements. Facilities of the Gamma Irradiation Building are operated by Phillips Petroleum Company under contract to the AEC. (Photograph supplied by Phillips Petroleum Co., Idaho Falls, Idaho.)

an atomic nucleus through radioactive decay. The establishment of proper dosage levels for food irradiation is important. Currently four ranges of radiation are of interest. A relatively low dose of

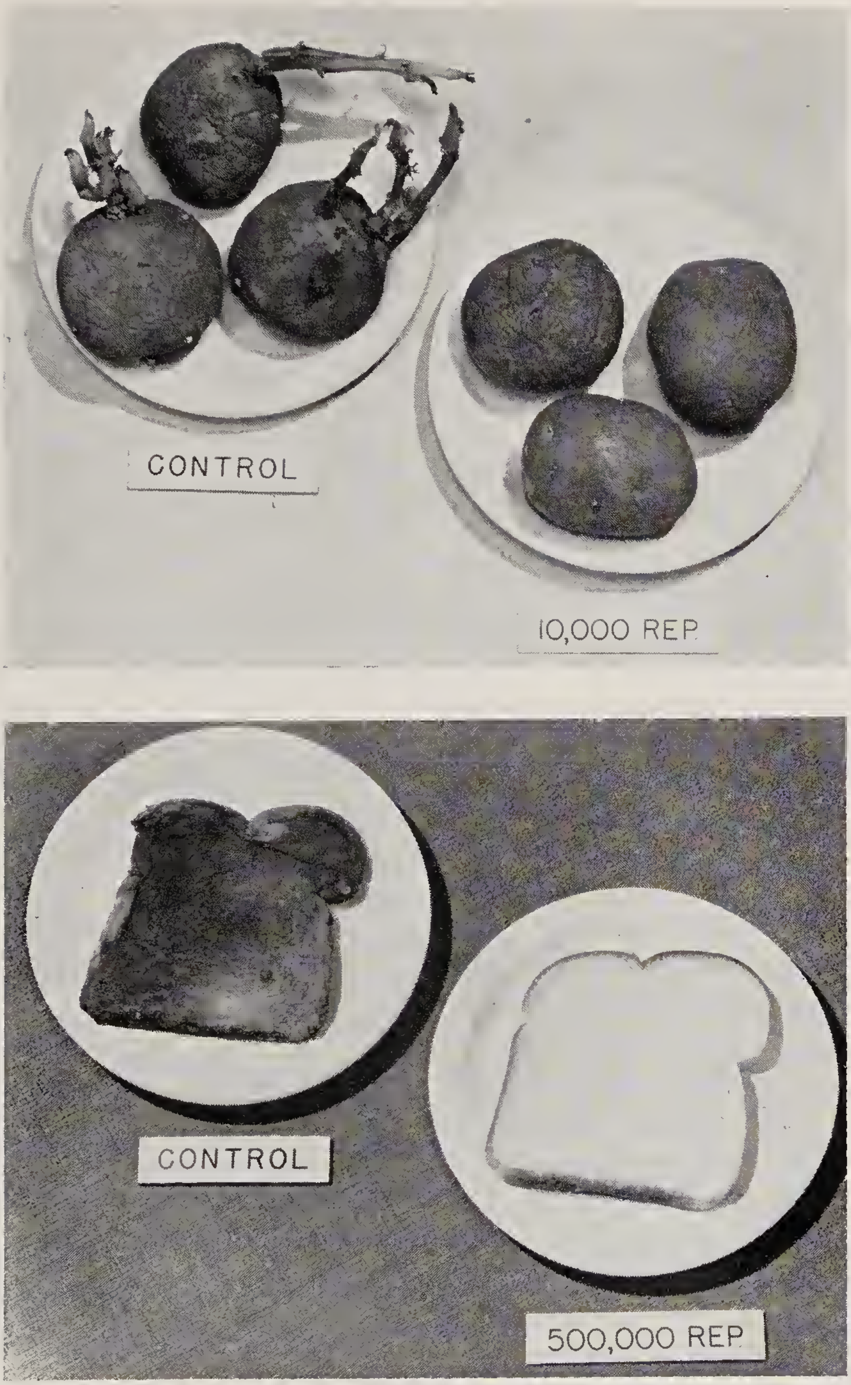


Fig. 2. Application of radiation to extend the storage life of foods; *top*, potatoes irradiated with a dosage of $10^4$ rep, *bottom*, bread irradiated with $5 \times 10^5$ rep. Experimental data: *potatoes* harvested Oct. 1954, irradiated Jan. 26, 1955, photographed Apr. 26, 1955, stored at 45°F. until Mar. 9 and at 55–65°F. until Apr. 26, $\gamma$-radiation source, Univ. of Michigan; *bread* irradiated Feb. 20, 1955, photographed Apr. 20, 1955, stored at room temperature, radiation source 1 MEV G.E. resonant transformer, Swift & Co. (Photographs supplied by the Quartermaster Food & Container Institute for the Armed Forces, Chicago, Ill.)

about $10^4$ roentgen equivalent physical (rep)[1] has been found useful in treatment of potatoes (Fig. 2) and onions for sprout inhibition and in treatment of grain for insect control. Dosages about 10 times higher have been used for radiopasteurization of refrigerated fresh meats and other foods (Fig. 2) which are to be consumed relatively soon. The term radiopasteurization has sometimes been referred to as cold pasteurization and is applied to those food radiation processes which decrease but do not entirely eliminate bacteria (e.g., dosages of about $7 \times 10^4$ rep). A dosage of about $10^6$ rep has been used for more intense radiopasteurization in which all vegetative organisms are destroyed and where the food is to be held under refrigeration for several weeks. The highest useful radiation dose is from 2 to 4 million rep; this is a sterilizing dose which might be used on canned foods stored at room temperature.

The question of what happens to irradiated foods is an interesting one. It was announced in the year 1905 that electromagnetic radiations of the type discovered by Becquerel and Roentgen would destroy bacteria. Until recently it was believed that ionizing particles killed by colliding with bacteria, producing free oxidative and reductive radicals which killed the cell either through alteration of its surface chemistry or destruction of its biochemical equilibrium (79,180). The direct hit theory, which probably accounts for most of the destruction of bacteria, implies that the medium in which the bacteria are living should have no effect on the rate of destruction by radiation. Recent work has indicated that this may not be entirely correct. Some bacteria apparently are killed by indirect action of the ionizing radiations on the medium. Perhaps some bacteria are killed by free radicals produced outside the bacterial cell which react chemically with the bacterial surface or penetrate the cell wall. In a complex medium such as meat, some of the free radicals have a better chance of reacting with other components of the medium and are inactivated before they can react with the bacteria. Additional evidence for this indirect bactericidal effect comes from experiments in which greater destruction of bacteria occurred following irradiation of milk at about 40°F. than when the milk was frozen and irradiated. Mobility of the free radicals believed to be responsible for the destruction of bacteria by the indirect effect was apparently reduced when the irradiated medium was frozen. It has also been found that *Escherichia coli* are more sensitive to radiation in the presence of oxygen than in its absence. The radiosensitivity of *E. coli* is affected also by environmental conditions during development and growth of the bacteria prior to radiation. The potential implications of the indirect effect of radiation on bacteria have prompted investigations of environmental factors such as temperature, pH, and chemical additives to the bacterial medium (8,34,35,117,218,269,270). The action of ionizing radiation on bacteria is also dependent upon the species involved.

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[1] The roentgen unit (r.) is that quantity of x- or gamma-radiation such that the associated corpuscular emission per 0.001293 g. of air produces, in air, ions carrying 1 electrostatic unit of quantity of electricity of either sign. If the energy lost by ionization in tissues is the same as the energy loss for 1 r. of gamma-radiation absorbed in air, the dose is spoken of as 1 roentgen-equivalent-physical (rep).

The largest problem to be solved before irradiation can be used in food processing is the elimination of undesirable side reactions which result in off-flavor and other properties that reduce consumer acceptability (29,82,112,115,127,148,201). Although there is little or no loss of essential nutrients in some foods subjected to sterilizing levels of irradiation, there are often large differences in flavor, odor, and sometimes color and texture of foods irradiated at levels below those necessary for complete sterilization. Thus, while the degree of change in a particular nutrient in an irradiated food is so small as to be beyond the accuracy of modern chemical instrumentation, it is sufficiently great to be detected easily by human smell and taste (115). Even though these changes are quantitatively very small, they are of great qualitative importance, not only with regard to flavor and odor but also in considering the possibilities of a toxic product resulting from modification of a nutrient or vitamin. The Food and Drug Administration has indicated its position on this point and had outlined animal feeding tests for the evaluation of a given commodity in this respect (181). Wasserman and Trum (294) found no adverse effects after feeding dogs a diet consisting largely of meat from lethally irradiated cattle and sheep. Studies at the University of Michigan produced no evidence that $\gamma$-irradiated diets were unwholesome, aside from a certain amount of vitamin destruction. Work at the U. S. Army's Medical Nutrition Laboratory, Swift & Co., Columbia University, and Massachusetts Institute of Technology has indicated no gross toxicity of irradiated products; vitamin destruction was of the same magnitude as that in heat-processed foods. Additional consideration is being given to acute and chronic feeding experiments with several animals and humans, tests for carcinogenicity, and effects on nutrients (10).

The type and quantity of irradiation for certain foods has been established. On a weight basis the potato is the most important single vegetable in the domestic diet. About 75% of the U. S. potato crop is harvested in the fall and cannot be held in unrefrigerated storage later than early spring. With refrigeration, potatoes can be held in storage for a maximum of about 8 months. Sparrow and Christensen (268) have shown that ionizing radiations successfully inhibited sprouting of potatoes at low doses (e.g., $2 \times 10^4$ r.) and after 18 months of storage the potatoes displayed little or no loss in quality. Evans (87) has reported similarly. Kuhl *et al.* (176) have designed a portable pilot plant using $Co^{60}$ for irradiating potatoes, and Starr and Cinnamon (271) have suggested further tests for the control of ring-rot bacteria, *Corynebacterium sepedonicum,* infesting burlap potato bags. A commercial processing plant for irradiation of potatoes has been described (9). Dallyn *et al.* (74) have also extended the storage life of onions by irradiation. The irradiation of butter fat (127) and vitamin and enzyme retention in milk and related products coincident with irradiation (148) have also been studied. The threshold value for recognition of off-flavors due to irradiation is about $2 \times 10^4$ rep for skim milk and $7 \times 10^3$ rep for whole milk. To guarantee complete sterilization of milk under adverse conditions, a dosage of at least $2 \times 10^6$ rep is required. Therefore the extremely low threshold dose for milk off-flavors presents a problem which must be solved before this method can have commercial value. Gomberg *et al.* (118,119,120,121,122,124) have successfully eliminated trichinella in pork by exposures of $7.5 \times 10^5$ to $1 \times 10^6$ rep; about $2 \times 10^4$ rep of $\gamma$-radiation prevents development of the larvae into adults and $1.2 \text{ x } 10^4$ rep completely sterilizes the female worms. These procedures control trichinosis without high radiation cost or deleterious flavor changes. Gould (123) has reviewed the effects of roentgen and other irradiation treatments of trichinosis in rats and mice.

A number of compounds with objectionable odors are produced in meat, especially ground fresh beef, subjected to high energy radiation and methods are needed to improve the acceptability of these irradiated products. Among compounds detected in irradiated meat are hydrogen sulfides, methyl mercaptan, carbonyl compounds, alkyl sulfides, and alkyl aldehydes. In the case of meats, it appears that surface sterilization (radiopasteurization) with dosages of less than $10^5$ rep offers considerably more promise for early commercial application than does radiation sterilization using higher dosages. The economic feasibility and commercial potentialities of food preservation by radiation effects have been discussed by Brownell (53) and Clifcorn (65).

Cooperative research between the Brookhaven National Laboratory and the U. S. Department of Agriculture Nematode Research Laboratory has shown that relatively small doses of radiation will produce sterility in nematodes. Nematode infections are very serious in crops such as potatoes and strawberries. By irradiating potatoes from an infected area, it might be possible to prevent the spread of the infestation as well as prolong the storage life of the potatoes by preventing them from sprouting, as mentioned earlier in this chapter. Nematode infections in strawberries are usually passed along with new plants; by irradiating the strawberry plants before planting, it might be possible to maintain a field free of infection. Preliminary investigations along these lines are very encouraging and warrant further study.

Radiation effects have been used successfully to control several insect pests of stored products. Brownell *et al.* (54) at the University of Michigan have designed a gamma ray radiation facility which may serve as a prototype for commercial insect control installations. The cost of properly irradiating flour with gamma rays would be in the same range as the cost of chemical control methods. Gamma radiation would be supplied by cooling fuel elements from a nuclear reactor. Although currently available in limited quantity, these fuel elements are expected to become much more plentiful as greater numbers of reactors are built in the future. The flour radiation facility has been designed for a radiation dose of $2.5 \times 10^4$ rep. This provides an adequate safety factor since a dose of only $10^4$ rep will sterilize the eggs of the confused flour beetle, *Tribolium confusum*, and the granary weevil, *Sitophilus granarius*, and prevent the adults from reproducing. An advantage of gamma radiation over chemical fumigation is the lethal effect of radiation on insect eggs as well as on adult insects. However, the irradiation process has no residual effect and therefore would not prevent recontamination of treated products from the outside if they were not suitably protected.

Although the level of radiation used did not appear to harm the flour, bread loaves made from irradiated flour were slightly smaller than those made with nonirradiated flour.

Hassett and Jenkins (135) also have controlled insect infestations in finished and packaged products with fission-product gamma rays. In tests with $Co^{60}$ and 6 species of stored products insect pests, reproduction was inhibited with $1.6$ to $3.2 \times 10^4$ r.; $6.5 \times 10^4$ r. doses were completely fatal. For insect control purposes, doses of lesser intensity stop reproduction and control light infestations, heavier doses quickly stop the damage of heavy infestations. The amount of $\gamma$-radiation necessary for killing insects is much less than is needed for bacterial sterilization (e.g., $10^6$ r.) but far more than lethal doses for mammals. Extensive insect damage to material can be prevented without excessive exposure to radiation. Extremely high doses may produce flavor changes in the exposed products. Packaging some products prior to treatment would prevent infestation by those insects unable to penetrate the container. Baker *et al.* (21,22) used a Van de Graaff accelerator to determine the lethal effects of electrons on insects which infest wheat, flour, and beans. An electron dose of $10^4$ rep sterilized eggs of the confused flour beetle and the granary weevil; the same dose prevented the adults from reproducing. A dose of $5 \times 10^5$ rep was lethal to adults of the same two insects. Wheat was damaged by a dose of $10^5$ rep; $10^6$ rep allowed the wheat to germinate but inhibited further growth. A slight change was detected in the taste of bread made from irradiated flour. An infestation of bean weevils, *Acanthoscelides obtectus,* in navy beans was arrested by a dose of $10^5$ rep. Full-grown Mediterranean flour moth larvae, *Anagasta kühniella,* failed to pupate following irradiation with x-ray doses ranging from 4 to $16 \times 10^4$ r. The behavior of the irradiated larvae resembled that of larvae in diapause and suggested a prolonged arrest of growth in this stage (301).

The release of large numbers of irradiated, sexually sterile pupae of the screw-worm, *Callitroga hominivorax,* gives rise to sterile male flies which mate with native females. The resulting eggs are infertile. If sterile pupae are released in sufficient numbers over a long enough period of time, the existing natural screw-worm population in the area is greatly reduced or virtually eliminated (30,175,185). The Entomology Research Branch, U. S. Department of Agriculture, has developed procedures for sterilizing large numbers of screw-worm pupae with $\gamma$-radiation from $Co^{60}$ for use in field experiments (57,75).

Atomic radiation has been used to speed the evolution process in plants and thereby offer the promise of creating new varieties of plants which can resist diseases (286) and destructive insects. This approach is essentially an extension of the work which has been going on for 3 decades using x-rays to increase genetic mutation rates. More versatile radiation sources used in connection with plant breeding may contribute to new methods of pest control. However, in the long run, natural evolution of new diseases and insect varieties may catch up with the new types of plants created through radiation effects. The effects of radiation on protozoa, viruses, bacteria, and fungi have been adequately discussed elsewhere (146).

## References

1. Abrams, R., *J. Am. Chem. Soc.*, *71*, 3835 (1949).
2. Acree, F., Jr., and Babers, F., *Science*, *120*, 948 (1954).
3. Acree, F., Jr., and Babers, F., *J. Econ. Entomol.*, *49*, 135 (1956).
4. Acree, F., Jr., Roan, C., and Babers, F., *J. Econ. Entomol.*, *47*, 1066 (1954).
5. Adams, P., and Kritchevsky, D., *Univ. Calif. Radiation Lab., Berkeley*, 1262 (1951).
6. Ahmed, M., Newsom, L., Emerson, R., and Roussel, J., *J. Econ. Entomol.*, *47*, 445 (1954).
7. Ahmed, M., Newsom, L., Roussel, J., and Emerson, R., *J. Econ. Entomol.*, *47*, 684 (1954).
8. Anderson, E., and Billen, D., *J. Bacteriol.*, *70*, 35 (1955).
9. Anonymous, *J. Agr. Food Chem.*, *2*, 1265 (1954).
10. Anonymous, *J. Agr. Food Chem.*, *4*, 107 (1956).
11. Aragão, M., *Rev. brasil. malariol. e doenças. trop.*, *5*, 137 (1953).
12. Aragão, M., Frota-Pessôa, E., and Margem, N., *Rev. brasil. malariol. e doenças. trop.*, *5*, 69 (1953).
13. Aragão, M., Frota-Pessôa, E., and Margem, N., *Proc. Intern. Conf. Peaceful Uses of Atomic Energy*, United Nations, New York, *10*, 525 (1956).
14. Arnason, A., Fuller, R., and Spinks, J., *Science*, *111*, 5 (1950).
15. Arnason, T., Irwin, R., and Spinks, J., *Can. J. Research* (*D.*), *27*, 186 (1949).
16. Arnason, T., Irwin, R., and Spinks, J., *Can. J. Zool.*, *29*, 234 (1951).
17. Aronoff, S., *Techniques of Radiobiochemistry*, Iowa State College Press, Ames, Ia., 1956.
18. Babers, F., and Roan, C., *J. Econ. Entomol.*, *47*, 973 (1954).
19. Babers, F., Roan, C., and Walker, R., *J. Econ. Entomol.*, *47*, 928 (1954).
20. Bacq, Z., and Alexander, P., *Fundamentals of Radiobiology*, Academic Press, Inc., New York, 1955.
21. Baker, V., Taboada, O., and Wiant, D., *Agr. Eng.*, *34*, 755 (1953).
22. Baker, V., Taboada, O., and Wiant, D., *Agr. Eng.*, *35*, 407 (1954).
23. Baldwin, R., Freed, V., and Fang, S., *J. Agr. Food Chem.*, *2*, 428 (1954).

24. Banks, C., *Brit. J. Animal Behaviour, 2*, 37 (1954).
25. Banks, C., *Brit. J. Animal Behaviour, 3*, 158 (1955).
26. Barker, D., and Christian, J., *J. Am. Pharm. Assoc., Sci. Ed., 44*, 105 (1955).
27. Bateman, A., and Sinclair, W., *Nature, 165*, 117 (1950).
28. Batt, R., Bennett, S., and Thomas, W., *Ann. Appl Biol., 41*, 475 (1954).
29. Batzer, O., and Doty, D., *J. Agr. Food Chem., 3*, 64 (1955).
30. Baumhover, A., Graham, A., Bitter, B., Hopkins, D., New, W., Dudley, F., and Bushland, R., *J. Econ. Entomol., 48*, 462 (1955).
31. Beamer, W., *J. Am. Chem. Soc., 70*, 3900 (1948).
32. Belleau, B., and Heard, R., *J. Am. Chem. Soc., 72*, 4268 (1950).
33. Bennett, S., and Thomas, W., *Ann. Appl. Biol., 41*, 484 (1954).
34. Beutler, E., Robson, M., and Jacobson, L., *Proc. Soc. Exptl. Biol. Med., 85*, 682 (1954).
35. Billen, D., Stapleton, G., and Hollaender, A., *J. Bacteriol., 65*, 131 (1953).
36. Bjorling, K., Lihnell, D., and Ossiannilsson, F., *Acta Agr. Scand., 1*, 301 (1951).
37. Blumel, J., *Science, 111*, 205 (1950).
38. Bowen, V., *Proc. Brookhaven Conf. Biological Application Nuclear Physics, BNL-C-4*, 104 (1948).
39. Bowen, V., *Trans. N. Y. Acad. Sci.*, Series II, *11*, 68 (1949).
40. Bowen, V., *J. Exptl. Zool., 115*, 175 (1950).
41. Bowen, V., Rubinson, A., and Sutton, D., *J. Exptl. Zool., 118*, 509 (1951).
42. Boyd, G., *Autoradiography in Biology and Medicine*, Academic Press Inc., New York, 1955.
43. Bradbury, F., Nield, P., and Newman, J., *Nature, 172*, 1052 (1953).
44. Bradbury, F., and Standen, H., *J. Sci. Food Agr., 6*, 90 (1955).
45. Bradbury, F., and Whitaker, W., *J. Sci. Food Agr., 7*, 248 (1956).
46. Brandes, G., Rohm & Haas Company, Philadelphia 5, Pa., personal communication, May 15, 1956.
47. Brauer, R., *J. Pharmacol. Exptl. Therap., 92*, 162 (1948).
48. Brauer, R., and Pessotti, R., *Federation Proc., 8*, 276 (1949).
49. Brauer, R., and Pessotti, R., *Science, 110*, 395 (1949).
50. Brian, M., *J. Animal Ecol., 16*, 210 (1947).
51. Bridges, R., *J. Sci. Food Agr., 6*, 261 (1955).
52. Brown, S., and Byerrum, R., *J. Am. Chem. Soc., 74*, 1523 (1952).
53. Brownell, L., *Food Manuf., 28*, 383 (1953).
54. Brownell, L., Nehemias, J., and Bulmer, J., *AECU-3050*, Univ. Michigan, Ann Arbor, Mich. (1955).
55. Bugher, J., and Taylor, M., *Science, 110*, 146 (1949).
56. Buhler, D., Wang, C., and Christensen, B., *J. Am. Chem. Soc., 75*, 4336 (1953).
57. Bushland, R., and Hopkins, D., *J. Econ. Entomol., 46*, 648 (1953).
58. Butts, J., Chang, S., Christensen, B., and Wang, C., *Science, 117*, 699 (1953).
59. Calvin, M., Heidelberger, C., Reid, J., Tolbert, B., and Yankwich, P., *Isotopic Carbon, Techniques in Its Measurement and Chemical Manipulation*, Wiley, New York, 1949.

60. Campbell, F., and Lukens, C., *J. Econ. Entomol.*, *24*, 88 (1931).
61. Carter, W., *J. Econ. Entomol.*, *38*, 335 (1945).
62. Casida, J., Gatterdam, P., Getzin, L., Jr., and Chapman, R., *J. Agr. Food Chem.*, *4*, 236 (1956).
63. Chatters, R., *A Summary of Research on a* $P^{32}$*- and* $S^{35}$*-Labelled Trialkyl Thiophosphate Systemic Insecticide, Systox.* Oklahoma A & M College, 5 p. mimeo., March 23, 1953.
64. Christenson, F., and Tolbert, B., *AECU-373* (1949).
65. Clifcorn, L., *Nucleonics*, *13* (1), 39 (1955).
66. Collier, P., *Chemistry & Industry*, *51*, 1122 (1951).
67. Comar, C., *Radioisotopes in Biology and Agriculture*, McGraw-Hill, New York, 1955.
68. Cornwell, P., *Nature*, *175*, 85 (1955).
69. Cox, J., and Warne, R., *J. Chem. Soc.*, *1951*, 1893.
70. Craig, J., Tryon, P., and Brown, W., *Anal. Chem.*, *25*, 1661 (1953).
71. Craig, R., and Olson, N., *Science*, *113*, 648 (1951).
72. Dahm, P., *U. S. Atomic Energy Comm.*, *TID-5098* (1953).
73. Dahm, P., *Soap Sanit. Chemicals*, *29*(9), 136–7, 139, 161; (10), 148–9, 151, 153, 163, 165; (11), 141, 143, 145, 147, 165; (12), 167–8, 175 (1953).
74. Dallyn, S., Sawyer, R., and Sparrow, A., *Nucleonics*, *13* (4), 48 (1955).
75. Darden, E., Jr., Maeyens, E., and Bushland, R., *Nucleonics*, *12*(10), 60 (1954).
76. David, W., *Nature*, *166*, 72 (1950).
77. David, W., *Ann. Appl. Biol.*, *38*, 508 (1951).
78. David, W., *Ann. Appl. Biol.*, *39*, 203 (1952).
79. Davis, M., *Arch. Biochem. and Biophys.*, *48*, 469 (1954).
80. Dawson, R., Christman, D., and Anderson, R., *J. Am. Chem. Soc.*, *75*, 5114 (1953).
81. Day, M., and Irzykiewicz, H., *Australian J. Biol. Sci.*, *6*, 98 (1953).
82. Doty, D., and Wachter, J., *J. Agr. Food Chem.*, *3*, 61 (1955).
83. Ducoff, H., Neal, W., Straube, R., Jacobson, L., and Brues, A., *Proc. Soc. Exptl. Biol. Med.*, *69*, 548 (1948).
84. Dupée, L., Heath, D., and Otter, I., *J. Agr. Food Chem.*, *4*, 233 (1956).
85. Earle, N., Ph.D. thesis, Univ. Illinois, Urbana, Ill., 1952.
86. Englund, K., *J. Agr. Food Chem.*, *3*, 826 (1955).
87. Evans, J., *Atomics*, *6*, 47 (1955).
88. Fang, S., and Allen, D., *J. Econ. Entomol.*, *48*, 79 (1955).
89. Fang, S., Freed, V., Johnson, R., and Coffee, D., *J. Agr. Food Chem.*, *3*, 400 (1955).
90. Fernando, H., Roan, C., and Kearns, C., *Ann. Entomol. Soc. Amer.*, *44*, 551 (1951).
91. Fields, M., Gibbs, J., and Waltz, D., *Science*, *112*, 591 (1950).
92. Fields, M., Rothchild, S., and Leaffer, M., *J. Am. Chem. Soc.*, *74*, 2435 (1952).
93. Flemion, F., Miller, L., and Weed, R., *Contribs. Boyce Thompson Inst.*, *16*, 429 (1952).

94. Flemion, F., Weed, R., and Miller, L., *Contribs. Boyce Thompson Inst., 16*, 285 (1951).
95. Foott, W., *Ann. Rept. Entomol. Soc. Ontario, 85*, 56 (1954).
96. Foreman, W., Murray, A., III, and Ronzio, A., *J. Org. Chem., 15*, 119 (1950).
97. Francis, G., Mulligan, W., and Wormall, A., *Isotopic Tracers. A Theoretical and Practical Manual for Biological Students and Research Workers*, The Athlone Press, London, 1954.
98. Fredeen, F., Spinks, J., Anderson, J., Arnason, A., and Rempel, J., *Can. J. Zool., 31*, 1 (1953).
99. Fredericksen, C., and Lilly, J., *J. Econ. Entomol., 48*, 438 (1955).
100. Friedlander, G., and Kennedy, J., *Nuclear and Radiochemistry*, Wiley, New York, 1955.
101. Fukuto, T., and Metcalf, R., *J. Am. Chem. Soc., 76*, 5103 (1954).
102. Fukuto, T., Metcalf, R., March, R., and Maxon, M., *J. Econ. Entomol., 48*, 347 (1955).
103. Fukuto, T., Wolf, J., III, Metcalf, R., and March, R., *J. Econ. Entomol., 49*, 147 (1956).
104. Fuller, R., Riegert, P., and Spinks, J., *Can. Entomologist, 86*, 201 (1954).
105. Fuller, R., Spinks, J., Arnason, A., and McDonald, H., *Ann. Rept. Entomol. Soc. Ontario, 81st*, 7 (*1950*) (Pub. *1951*).
106. Ganz, A., Kelsey, F., and Geiling, E., *J. Pharmacol. Exptl. Therap., 103*, 209 (1951).
107. Ganz, A., Kelsey, F., and Geiling, E., *Botan. Gaz., 113*, 195 (1951).
108. Gar, K., and Kipiani, R., *Proc. Intern. Conf. Peaceful Uses of Atomic Energy*, United Nations, New York, *12*, 185 (1956).
109. Gardiner, J., and Kilby, B., *Research, 2*, 590 (1949).
110. Gardiner, J., and Kilby, B., *J. Chem. Soc., 1950*, 1769.
111. Gardner, K., and Heath, D., *Anal. Chem., 25*, 1849 (1953).
112. Ginger, I., and Schweigert, B., *J. Agr. Food Chem., 4*, 885 (1956).
113. Gjullin, C., Lindquist, A., and Butts, J., *Mosquito News, 12*, 201 (1952).
114. Glynne Jones, G., and Thomas, W., *Ann. Appl. Biol., 40*, 546 (1953).
115. Goldblith, S., and Proctor, B., *J. Agr. Food Chem., 3*, 253 (1955).
116. Goldblith, S., Proctor, B., Davison, S., Kan, B., Bates, C., Oberle, E., Karel, M., and Long, D., *Food Research, 18*, 659 (1953).
117. Goldblith, S., Proctor, B., Davison, S., Oberle, E., Bates, C., Kan, B., Hammerle, O., and Kusmierek, B., *Nucleonics, 13*(1), 42 (1955).
118. Gomberg, H., and Gould, S., *Science, 118*, 75 (1953).
119. Gomberg, H., Gould, S., Nehemias, J., and Brownell, L., *Nucleonics, 12*(5), 38 (1954).
120. Gomberg, H., Gould, S., Nehemias, J., and Brownell, L., *Nucleonics, 12*(9), 66 (1954).
121. Gomberg, H., Gould, S., Nehemias, J., and Brownell, L., *Nuclear Engineering, Part III, Chem. Eng. Progr. Symposium Ser. No. 13*, 89 (1954).
122. Gomberg, H., Gould, S., Nehemias, J., and Brownell, L., *Food Eng., 26*, Part 2 (9), 78–80, 154–156 (1954).
123. Gould, S., *Trichinosis*, Chas. C Thomas, Springfield, Ill., 1945.

124. Gould, S., Gomberg, H., and Bethell, F., *Am. J. Public Health, 43,* 1550 (1954).
125. Gray, R., *Science, 115,* 129 (1952).
126. Hamilton, M., *Ann. Appl. Biol., 22,* 243 (1935).
127. Hannan, R., and Shepherd, H., *Nature, 170,* 1021 (1952).
128. Hansen, E., Hansen, J., and Craig, R., *J. Econ. Entomol., 37,* 853 (1944).
129. Hart, E., Fleischer, J., and Marrazzi, A., *Federation Proc., 8,* 300 (1949).
130. Hartley, G., *World Crops, 4,* 397 (1952).
131. Hartley, G., and Heath, D., *Nature, 167,* 816 (1951).
132. Hartley, G., Heath, D., Hulme, J., Pound, D., and Whittaker, M., *J. Sci. Food Agr., 2,* 303 (1951).
133. Hassett, C., and Jenkins, D., *Science, 110,* 109 (1949).
134. Hassett, C., and Jenkins, D., *Physiol. Zoöl., 24,* 257 (1951).
135. Hassett, C., and Jenkins, D., *Nucleonics, 10*(12), 42 (1952).
136. Heath, D., *Congr. intern. Phytopharm. III*$^e$ *Congr., Paris, 1954.*
137. Heath, D., Cleugh, J., Otter, I., and Park, P., *J. Agr. Food Chem., 4,* 230 (1956).
138. Heath, D., Lane, D., and Llewellyn, M., *J. Sci. Food Agr., 3,* 60 (1952).
139. Heath, D., Lane, D., and Llewellyn, M., *J. Sci. Food Agr., 3,* 69 (1952).
140. Hein, R., and McFarland, R., *J. Am. Chem. Soc., 74,* 1856 (1952).
141. Hevesy, G., *Radioactive Indicators,* Interscience Publishers, New York, 1948.
142. Hill, R., Jones, A., and Palin, D., *Chemistry & Industry, 6,* 162 (1954).
143. Hoffman, R., Lindquist, A., and Butts, J., *J. Econ. Entomol., 44,* 471 (1951).
144. Hoffman, R., Roth, A., and Lindquist, A., *J. Econ. Entomol., 44,* 734 (1951).
145. Hoffman, R., Roth, A., Lindquist, A., and Butts, J., *Science, 115,* 312 (1952).
146. Hollaender, A., *Radiation Biology, Vol. 2, Ultraviolet and Related Radiations,* McGraw-Hill, New York, 1955.
147. Holley, R., Boyle, F., and Hand, D., *Arch. Biochem., 27,* 143 (1950).
148. Kung, Hung-Chun, Gaden, E., Jr., and King, C., *J. Agr. Food Chem., 1,* 142 (1953).
149. Irwin, R., Spinks, J., and Arnason, T., *Can. J. Research (D.), 28,* 137 (1950).
150. Jackson, P., and Hopkins, H., *U. S. Dept. Agr., Bur. Plant Industry, Div. Soil Management and Irrigation, Research Rept. No. 237* (1952).
151. Jandorf, B., and McNamara, P., *Federation Proc., 8,* 210 (1949).
152. Jandorf, B., and McNamara, P., *J. Pharmacol. Exptl. Therap., 98,* 77 (1950).
153. Jansen, E., Nutting, M.-D. Fellows, Jang, R., and Balls, A., *J. Biol. Chem., 179,* 201 (1949).
154. Jaworski, E., and Butts, J., *Arch. Biochem. and Biophys., 38,* 207 (1952).
155. Jenkins, D., *J. Econ. Entomol., 42,* 988 (1949).
156. Jenkins, D., *Exptl. Parasitol., 3,* 474 (1954).
157. Jenkins, D., *Proc. Intl. Conf. Peaceful Uses of Atomic Energy,* United Nations, New York, *10,* 518 (1956).
158. Jenkins, D., and Hassett, C., *Nucleonics, 6*(3), 5 (1950).
159. Jenkins, D., and Hassett, C., *Can. J. Zool., 29,* 178 (1951).
160. Jensen, J., Durham, W., and Pearce, G., *Arch. Ind. Hyg. Occupational Med., 6,* 326 (1952).
161. Jensen, J., and Fay, R., *Am. J. Trop. Med., 31,* 523 (1951).

162. Jensen, J., and Pearce, G., *J. Am. Chem. Soc.*, *74*, 2436 (1952).
163. Jensen, J., and Pearce, G., *J. Am. Chem. Soc.*, *74*, 3184 (1952).
164. Johnson, R., and Huston, J., *J. Am. Chem. Soc.*, *72*, 1841 (1950).
165. Jones, R., *J. Chem. Soc.*, *1952*, 4080.
166. Kamen, M., *Radioactive Tracers in Biology. An Introduction to Tracer Methodology*, 2nd ed., Academic Press, New York, 1951.
167. Kemp, H., Greenlee, R., Baldwin, M., Davidson, R., and Ellis, W., Jr., *AECU-3117* (1955).
168. Kettlewell, H., *Nature*, *170*, 584 (1952).
169. Kettlewell, H., *Nature*, *175*, 821 (1955).
170. Kettlewell, H., *Proc. Intl. Conf. Peaceful Uses of Atomic Energy*, United Nations, New York, *12*, 214 (1956).
171. Kilby, B., *Chemistry & Industry*, *1953*, 856.
172. King, R., and Wilson, L., *J. Exptl. Zool.*, *126*, 401 (1954).
173. Kleiber, M., Smith, A., and Ralston, N., *Proc. Soc. Exptl. Biol. Med.*, *69*, 354 (1948).
174. Knapp, S., Farinacci, C., Herbert, C., Jr., and Saenger, E., *J. Econ. Entomol.*, *49*, 393 (1956).
175. Knipling, E., *J. Econ. Entomol.*, *48*, 459 (1955).
176. Kuhl, O., Sparrow, A., and Manowitz, B., *Nucleonics*, *13*(11), 128 (1955).
177. Langston, R., *J. Agr. Food Chem.*, *3*, 849 (1955).
178. Lanz, H., Jr., Wallace, P., and Hamilton, J., *Univ. Calif. (Berkeley) Publs. Pharmacol.*, *2*, 263 (1949).
179. Lawson, F., Lucas, G., and Hall, N., *J. Econ. Entomol.*, *47*, 749 (1954).
180. Lea, D., *Actions of Radiations on Living Cells*, Macmillan, New York, 1947.
181. Lehman, A., and Laug, E., *Nucleonics*, *12*(1), 52 (1954).
182. LeRoux, E., and Morrison, F., *J. Econ. Entomol.*, *46*, 1109 (1953).
183. LeRoux, E., and Morrison, F., *J. Econ. Entomol.*, *47*, 1058 (1954).
184. Lindquist, A., *J. Econ. Entomol.*, *45*, 264 (1952).
185. Lindquist, A., *J. Econ. Entomol.*, *48*, 467 (1955).
186. Lindquist, A., Roth, A., Hoffman, R., and Butts, J., *J. Econ. Entomol.*, *44*, 931 (1951).
187. Lindquist, A., Roth, A., Yates, W., Hoffman, R., and Butts, J., *J. Econ. Entomol.*, *44*, 167 (1951).
188. Lindquist, A., Yates, W., Hoffman, R., and Butts, J., *J. Econ. Entomol.*, *44*. 397 (1951).
189. Lindquist, D., and Dahm, P., *J. Econ. Entomol.*, *49*, 579 (1956).
190. Lindsay, E., and Craig, R., *Ann. Entomol. Soc. Amer.*, *35*, 50 (1942).
191. Lockau, S., and Lüdicke, M., *Z. Naturforsch*, *7b*, 389 (1952).
192. Lockau, S., Lüdicke, M., and Weygand, F., *Naturwissenschaften*, *38*, 350 (1951).
193. Loftfield, R., *Nucleonics*, *1*(3), 54 (1947).
194. Logan, A., and Murray, J., *J. Am. Chem. Soc.*, *74*, 2436 (1952).
195. Low, A., *Chemistry & Industry*, *51*, 1124 (1951).
196. McCallan, S., and Miller, L., *Agr. Chemicals*, *9*(2), 52, 53, 119, 121 (1954).
197. McCarter, J., *J. Am. Chem. Soc.*, *73*, 483 (1951).

198. McCollister, D., Beamer, W., Atchison, G., and Spencer, H., *J. Pharmacol. Exptl. Therap.*, *102*, 112 (1951).
199. MacDonald, R., and Baker, B., *Can. J. Chem.*, *31*, 517 (1953).
200. Mahler, H., Speer, R., and Roberts, A., *Science*, *110*, 562 (1949).
201. Marbach, E., and Doty, D., *J. Agr. Food Chem.*, *4*, 881 (1956).
202. March, R., Fukuto, T., Metcalf, R., and Maxon, M., *J. Econ. Entomol.*, *49*, 185 (1956).
203. March, R., Metcalf, R., and Fukuto, T., *J. Agr. Food Chem.*, *2*, 732 (1954).
204. March, R., Metcalf, R., Fukuto, T., and Gunther, F., *J. Econ. Entomol.*, *49*, 679 (1956).
205. March, R., Metcalf, R., Fukuto, T., and Maxon, M., *J. Econ. Entomol.*, *48*, 355 (1955).
206. Metcalf, R., *Agr. Chemicals*, *9*(3), 33–35, 128–130 (1954).
207. Metcalf, R., Fukuto, T., March, R., and Stafford, E., *J. Econ. Entomol.*, *49*, 738 (1956).
208. Metcalf, R., Fukuto, T., Reynolds, H., and March, R., *J. Agr. Food Chem.*, *3*, 1011 (1955).
209. Metcalf, R., and March, R., *J. Econ. Entomol.*, *45*, 988 (1952).
210. Metcalf, R., March, R., Fukuto, T., and Maxon, M., *J. Econ. Entomol.*, *47*, 1045 (1954).
211. Metcalf, R., March, R., Fukuto, T., and Maxon, M., *J. Econ. Entomol.*, *48*, 364 (1955).
212. Michel, H., and Krop, S., *Federation Proc.*, *8*, 320 (1949).
213. Miller, L., *U. S. Atomic Energy Comm.*, *T.I.D.*-7512 (1956).
214. Miller, L., and McCallan, S., *Proc. Intl. Conf. Peaceful Uses of Atomic Energy*, United Nations, New York, *12*, 170 (1956).
215. Miller, L., Weed, R., and Hitchcock, A., *Contribs. Boyce Thompson Inst.*, *17*, 397 (1954).
216. Mitchell, J., and Linder, P., *Science*, *112*, 54 (1950).
217. Mitchell, J., and Linder, P., *Botan. Gaz.*, *112*, 126 (1950).
218. Morgan, B., and Reed, J., *Food Research*, *19*, 357 (1954).
219. Morrison, F., and LeRoux, E., *Can. J. Agr. Sci.*, *34*, 316 (1954).
220. Morrison, F., and Oliver, W., *Can. J. Research* (*D.*), *27*, 265 (1949).
221. Mühlmann, R., and Tietz, H., *Höfchen-Briefe*, Bayer (Leverkusen, Germany) Pflanzenschutz-Nachrichten (English edition), *9*, 116 (1956).
222. Murray, D., and Spinks, J., *Can. J. Chem.*, *30*, 497 (1952).
223. Norton, L., and Hansberry, R., *J. Econ. Entomol.*, *34*, 431 (1941).
224. Oertel, E., Emerson, R., and Wheeler, H., *Ann. Entomol. Soc. Am.*, *46*, 596 (1953).
225. Oughton, J., *Ann. Rept. Entomol. Soc. Ontario*, *81*, 91 (1950).
226. Palin, D., *Anal. Chem.*, *26*, 1856 (1954).
227. Patton, R., and Craig, R., *J. Exptl. Zool.*, *81*, 437 (1939).
228. Pearce, G., and Jensen, J., *Science*, *118*, 45 (1953).
229. Pearce, G., and Jensen, J., *J. Agr. Food Chem.*, *1*, 776 (1953).
230. Pellegrini, J., Jr., Miller, A., and Sharpless, R., *J. Econ. Entomol.*, *45*, 532 (1952).

231. Pendleton, R., and Grundmann, A., *Ecology, 35,* 187 (1954).
232. Perry, A., Jensen, J., and Pearce, G., *J. Agr. Food Chem., 3,* 1008 (1955).
233. Pietri-Tonelli, P., and March, R., *J. Econ. Entomol., 47,* 902 (1954).
234. Pimentel, D., and Fay, R., *J. Econ. Entomol., 48,* 19 (1955).
235. Pletsch, D., *Montana Agr. Exp. Sta. Tech. Bull., 446,* 95 (1947).
236. Provost, M., *Mosquito News, 12,* 174 (1952).
237. Quarterman, K., Jensen, J., Mathis, W., and Smith, W., *J. Econ. Entomol., 48,* 30 (1955).
238. Quarterman, K., Kilpatrick, J., and Mathis, W., *J. Econ. Entomol., 47,* 413 (1954).
239. Quarterman, K., Mathis, W., and Kilpatrick, J., *J. Econ. Entomol., 47,* 405 (1954).
240. Radeleff, R., Bushland, R., and Hopkins, D., *J. Econ. Entomol., 45,* 509 (1952).
241. Rings, R., and Layne, G., Jr., *J. Econ. Entomol., 46,* 473 (1953).
242. Ripper, W., Greenslade, R., and Hartley, G., *J. Econ. Entomol., 44,* 448 (1951).
243. Roan, C., *J. Econ. Entomol., 45,* 826 (1952).
244. Roan, C., Fernando, H., and Kearns, C., *J. Econ. Entomol., 43,* 319 (1950).
245. Robbins, W., and Dahm, P., *J. Agr. Food Chem., 3,* 500 (1955).
246. Robbins, W., Hopkins, T., and Eddy, G., *J. Agr. Food Chem., 5,* July (1957).
247. Robbins, W., Hopkins, T., and Eddy, G., *J. Econ. Entomol., 49,* 801 (1956).
248. Robinson, J., *Can. J. Chem., 33,* 722 (1955).
249. Rodriguez, J., *J. Econ. Entomol., 47,* 514 (1954).
250. Roth, A., and Hoffman, R., *J. Econ. Entomol., 45,* 1091 (1952).
251. Roth, A., Lindquist, A., and Terriere, L., *J. Econ. Entomol., 46,* 127 (1953).
252. Sacks, J., and Sinex, F., *Arch. Biochem. and Biophys., 39,* 205 (1952).
253. Saunders, B., and Worthy, T., *Nature, 163,* 797 (1949).
254. Saunders, B., and Worthy, T., *J. Chem. Soc., 1950,* 1320–1322.
255. Schmidt, C., and Dahm, P., *J. Econ. Entomol., 49,* 729 (1956).
256. Schoof, H., and Mail, G., *J. Econ. Entomol., 46,* 258 (1953).
257. Schoof, H., and Siverly, R., *J. Econ. Entomol., 47,* 830 (1954).
258. Schoof, H., and Siverly, R., *Am. J. Trop. Med. Hyg., 3,* 367 (1954).
259. Schoof, H., Siverly, R., and Jensen, J., *J. Econ. Entomol., 45,* 675 (1952).
260. Selff, R., and Tolbert, B., *Univ. Calif., Radiation Lab., Berkeley,* 1299 (1951).
261. Shellenberger, J., Dahm, P., Farrell, E., Hein, R., McFarland, R., Milner, M., and Lloyd, N., *Assoc. Operative Millers, Bull.,* (Oct., 1953).
262. Shemanchuk, J., Spinks, J., and Fredeen, F., *Can. Entomologist, 85,* 269 (1953).
263. Shura-Bura, B., *Zool. Zhur., 31,* 410 (1952).
264. Siri, W., Dougherty, E., Tobias, C., Robertson, J., Dunn, R., and Weymouth, P., *Isotopic Tracers and Nuclear Radiations with Applications to Biology and Medicine,* McGraw-Hill, New York, 1949.
265. Smales, A., and Pate, B., *Anal. Chem., 24,* 717 (1952).
266. Smales, A., and Pate, B., *Analyst, 77,* 196 (1952).
267. Smith, A., and Bohart, R., *Calif. Agriculture, 10*(7), 11 (1956).
268. Sparrow, A., and Christensen, E., *Nucleonics, 12*(8), 16 (1954).

269. Stapleton, G., *J. Bacteriol.*, *70*, 357 (1955).
270. Stapleton, G., Sbarra, A., and Hollaender, A., *J. Bacteriol.*, *70*, 7 (1955).
271. Starr, G., and Cinnamon, C., *Phytopathology*, *43*, 439 (1953).
272. Stein, L., *J. S. African Chem. Inst.*, *7*, 120 (1954).
273. Stein, L., Alper, T., and Anderssen, E., *J. Sci. Food Agr.*, *3*, 31 (1952).
274. Stein, L., and Smith, A., *J. S. African Chem. Inst.*, *7*, 114 (1954).
275. Sternburg, J., Kearns, C., and Moorefield, H., *J. Agr. Food Chem.*, *2*, 1125 (1954).
276. Straube, R., Neal, W., Jr., Kelly, T., and Ducoff, H., *Proc. Soc. Exptl. Biol. Med.*, *69*, 270 (1948).
277. Strittmatter, C., Peters, T., Jr., and McKee, R., *Arch. Ind. Hyg. Occupational Med.*, *1*, 54 (1950).
278. Stutz, R., Atkinson, D., and Gordon, S., *Argonne Natl. Lab.*, 4710 (1951).
279. Terriere, L., and Schonbrod, R., *J. Econ. Entomol.*, *48*, 736 (1955).
280. Thomas, M., *Proc. Auburn Conf. on the Use of Radioactive Isotopes in Agricultural Research*, Edwards Brothers, Inc., Ann Arbor, Mich., 1948.
281. Thomas, W., and Bennett, S., *Ann. Appl. Biol.*, *41*, 501 (1954).
282. Thomas, W., Bennett, S., and Lloyd-Jones, C., *Ann. Appl. Biol.*, *43*, 569 (1955).
283. Thomas, W., and Glynne Jones, G., *Ann. Appl. Biol.*, *43*, 182 (1955).
284. Tietz, H., Höfchen-Briefe, Bayer (Leverkusen, Germany) Pflanzenschutz-Nachrichten (English edition), *7*, 1 (1954).
285. Tolbert, B., *J. Am. Chem. Soc.*, *69*, 1529 (1947).
286. Tolbert, N., and Pearson, P., *Atomic energy and the plant sciences. Advances in Agron.*, *4*, 279, 1952.
287. Tomes, G., and Brian, M., *Nature*, *158*, 551 (1946).
288. Tomisek, A., and Mahler, H., *J. Am. Chem. Soc.*, *73*, 4685 (1951).
289. Trenner, N., Walker, R., Arison, B., and Buhs, R., *Anal. Chem.*, *21*, 285 (1949).
290. Tsuyuki, H., Stahmann, M., and Casida, J., *J. Agr. Food Chem.*, *3*, 922 (1955).
291. Turrell, F., and Chervenak, M., *Botan. Gaz.*, *111*, 109 (1949).
292. Turrell, F., and Chervenak, M., *Advances in Chem. Ser., No. 1*, 250 (1950)
293. U. S. Atomic Energy Comm., *Isotopes, an Eight-Year Summary of Distribution and Utilization with Bibliography*, U. S. Atomic Energy Comm., Washington, D. C., 1955.
294. Wasserman, R., and Trum, B., *Science*, *121*, 894 (1955).
295. Watson, M., and Nixon, H., *Ann. Appl. Biol.*, *40*, 537 (1953).
296. Wedding, R., *J. Agr. Food Chem.*, *1*, 832 (1953).
297. Wedding, R., and Metcalf, R., *Botan. Gaz.*, *114*, 180 (1952).
298. West, A., and Jenkins, D., *Mosquito News*, *11*, 217 (1951).
299. Wheeler, B., *Proc. Natl. Acad. Sci. U. S.*, *33*, 298 (1947).
300. Wheeler, B., *J. Exptl. Zool.*, *115*, 83 (1950).
301. Whiting, A., *Anat. Record*, *108*, 609 (1950).
302. Winteringham, F., *J. Chem. Soc.*, *1949*, S416.
303. Winteringham, F., *Endeavour*, *11*, 22 (1952).
304. Winteringham, F., *Science*, *116*, 452 (1952).
305. Winteringham, F., *Bull. Natl. Research Council (U.S.)*, *Publ. 219*, 61 (1952).

306. Winteringham, F., *J. Sci. Food Agr.*, *6*, 269 (1955).
307. Winteringham, F., *Laboratory Practice*, *4*, 94–102, 148–154, 196–202, 244–250, 288–293, 328–333, 370–374, 411–415, 449–453, 493–496 (1955).
308. Winteringham, F., Harrison, A., and Bridges, R., *Nature*, *166*, 999 (1950).
309. Winteringham, F., Harrison, A., and Bridges, R., *Analyst*, *77*, 19 (1952).
310. Winteringham, F., Harrison, A., and Bridges, R., *Nucleonics*, *10*(3), 52 (1952).
311. Winteringham, F., Harrison, A., and Bridges, P., *Biochem. J.*, *61*, 359 (1955).
312. Winteringham, F., Harrison, A., Bridges, R., and Bridges, P., *J. Sci. Food Agr.*, *6*, 251 (1955).
313. Winteringham, F., Harrison, A., and Hammond, J., *Nature*, *165*, 149 (1950).
314. Winteringham, F., Harrison, A., Jones, C., McGirr, J., and Templeton, W., *J. Sci. Food Agr.*, *7*, 214 (1950).
315. Winteringham, F., and Hellyer, G., *Biochem. J.*, *58*, 45 (1954).
316. Winteringham, F., Loveday, P., and Harrison, A., *Nature*, *167*, 106 (1951).
317. Witten, B., and Miller, J., *J. Am. Chem. Soc.*, *70*, 3886 (1948).
318. Wolfe, W., Wood, J., Klipp, L., Fontaine, T., and Mitchell, J., *J. Org. Chem.*, *14*, 900 (1949).
319. Ya, A., Vladimirova, I., and Mel'nikov, N., Doklady Akad. Nauk. S.S.S.R. (in Russian), *100*, 77 (1955).
320. Yamafuji, K., and Omura, H., *Enzymologia*, *17*, 28 (1954).
321. Yates, W., Gjullin, C., Lindquist, A., and Butts, J., *J. Econ. Entomol.*, *44*, 34 (1951).
322. Yates, W., Lindquist, A., and Butts, J., *J. Econ. Entomol.*, *45*, 547 (1952).
323. Zeid, M., Dahm, P., Hein, R., and McFarland, R., *J. Econ. Entomol.*, *46*. 324 (1953).

# THE CHEMISTRY AND ACTION OF ORGANIC PHOSPHORUS INSECTICIDES

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## I. Introduction

·The use of organophosphorus compounds as insecticides during the past decade has led to the synthesis and insecticidal evaluation of thousands of many types of phosphorus-containing compounds. Although relatively few of those evaluated have attained commercial importance many are presently undergoing experimental evaluation and may prove to be superior as contact or stomach poisons, or as systemic insecticides, to those now available. Progress in the development of organophosphorus compounds as insecticides has been remarkably rapid since the findings of Gerhard Schrader and his group in Germany were published in 1947 (129). The cooperation between chemists, entomologists, and toxicologists has resulted in a clear understanding of some of the chemical reactions involved in the mode of action and metabolism of these toxic compounds in biological systems. Certain essential structural requisites necessary for insecticidal activity are also apparent from information obtained by the critical examination of the vast number of organophosphorus compounds. By making use of this knowledge and applying certain basic principles in chemical reactivity we are now in the enviable position to realize the development of new and better organic phosphorus insecticides.

The subject of the chemistry and biological action of organophosphorus compounds has reached such enormous stature that it is impossible to touch upon all phases of the subject with any degree of coverage within the pages of this presentation. It is the author's intention, therefore, to concentrate on some of the more fundamental aspects, and particular effort is made to correlate biological activity with known concepts in physical-organic chemistry. For a more detailed treatise of the subject of organic phosphate insecticides, the reader is referred to an excellent chapter in a recent book (101).

The mechanism by which the organic phosphorus insecticides exert their toxic actions on insects and mammals depends largely on the biochemical processes of the animal and the physico-chemical properties of the phosphorus compound. Fundamentally, however, the toxic symptoms produced in animals by these organophosphorus compounds are manifestations of the inhibition of certain enzyme systems. In order to fully understand the mechanism of action of these compounds it therefore becomes necessary to study the types and nature of the enzyme systems affected.

## II. The Nature of Enzymes Inhibited by Organic Phosphates

The organophosphorus compounds are inhibitors of a variety of esterases, a group of enzymes which catalyze the hydrolysis of esters. It is generally agreed that the toxicity to mammals is associated with the inhibition of the cholinesterase enzymes (ChE) whose normal

$$CH_3COOCH_2CH_2\overset{+}{N}(CH_3)_3 + H_2O \xrightarrow{AChE} CH_3COOH + HOCH_2CH_2\overset{+}{N}(CH_3)_3 \quad (1)$$

physiological function is to catalyze the hydrolysis of choline esters, exemplified by equation (1) which shows the hydrolysis of acetylcholine by acetylcholinesterase (AChE). Acetylcholine is believed to be the transmitting agent of nerve impulses to the effector cells at the neuromuscular junction (18) and its role in nerve action is discussed in several review articles and publications (18,115,116,52).

The results from numerous investigations concerning the specificity of enzymes hydrolyzing choline esters indicate the existence of at least two types: (*a*) *acetylcholinesterases* (AChE) (also referred to as true or specific cholinesterases) which show higher affinity for acetylcholine than any other ester tested and (*b*) cholinesterases (ChE) (also referred to as nonspecific or pseudo cholinesterases) which differ from AChE in activity-substrate concentration relationship (70) and in having a higher affinity for butyrylcholine than for acetylcholine (117,17,4). AChE may be distinguished from ChE in other ways in addition to the differences stated above. Acetyl-β-methylcholine is hydrolyzed by AChE and not by ChE; benzoylcholine is split only by the latter and not by AChE (98).

AChE is present in high concentration in nervous tissues of both vertebrates and invertebrates (107,117,99,128,86). The head of the common housefly (*Musca domestica* L.) contains large amounts of AChE and is used as a convenient source for the enzyme (107). The esterase present in the pigeon brain at a very high level (four times that of mammalian brain) is primarily AChE (142). AChE is present to some extent in muscular tissues (86,117) but in general the activities are low. A source of high activity AChE is from the electric organs of *Electrophorus electricus*, *Malapterurus electricus*, and *Torpedo marmorata* (117,114). The AChE of blood is usually located in erythrocytes. AChE is also found in the blood of *Helix pomatia* and in cobra venom (16,14) but these show certain differences in specificity patterns compared to the acetylcholinesterases discussed above. Blood ChE is located in the plasma of most animals but AChE and aliesterases are also present in varying amounts, e.g., horse and human serum contain ChE and aliesterases (1,132), whereas rabbit serum contains only AChE (2) and an esterase which is not affected by organophosphorus compounds.

In addition to the cholinesterases, other esterases are also affected by the organophosphorus compounds. Both trypsin and chymotrypsin are inhibited by di*iso*propyl phosphorofluoridate (DFP) and *O*,*O*-diethyl *O*-*p*-nitrophenyl phosphate (para-oxon) (78,68). Human liver esterase and milk lipase activity are strongly inhibited by DFP (141). The aliesterases of rat liver were found to be highly sensitive to inhibition by various organic phosphates such as para-oxon and DFP (112). The B-esterase or aliesterase present in various sera is strongly inhibited by para-oxon and other phosphates (2,111). Acetylesterase from oranges is inhibited by tetraethyl pyrophosphate (TEPP) and DFP (80).

Although many of the esterases are inhibited by the organic phosphates, a few are unaffected and some have been found to hydrolytically degrade certain organophosphorus compounds. The A-esterase found in the sera of the rabbit, rat, and horse is unaffected by para-oxon (2) and actually catalyzes its hydrolysis (5). An enzyme ("tabunase") present in the plasma and other tissues of the rabbit, man, horse, and several other animals was found to enzymatically hydrolyze ethyl dimethylphosphoramidocyanidate (tabun) and DFP to the corresponding acids (21,19,22). An aromatic esterase present in human erythrocytes and plasma capable of hydrolyzing a series of substituted-phenyl acetates and suggested to be identical with A-esterase is not affected by relatively high concentration of DFP or para-oxon (111). The aromatic esterase present in bee abdomen (*Apis mellifera*) is capable of hydrolyzing several organic phosphates including para-oxon. Little is known of the true function of these organic phosphate insensitive esterases but it is possible that they take part in the detoxification mechanism of these toxic compounds.

The toxicity of the organic phosphates to insects is also associated with the inhibition of the cholinesterase enzyme systems in those insects where they are regarded as physiologically important. The enzymatic make-up of various tissues of a number of insects have been studied in much detail and the presence of cholinesterase has been amply demonstrated in most orders of insects (33,97,127). Certain insect cholinesterases differ in some respects from the cholinesterase of mammals. The cholinesterase present in cockroach nerve cord, *Periplaneta americana*, and honey-bee, *Apis mellifera*, splits acetyl-β-methylcholine at a higher rate than acetylcholine (124,133,15). A more detailed study (25,107) showed that the cholinesterase of the honey-bee brain split acetyl-β-methylcholine faster than acetylcholine at high substrate concentration, the reverse being the case at low substrate concentration, whereas the cholinesterase of the housefly brain hydrolyzed acetylcholine faster at all concentrations. The cholinesterase of the housefly brain may be regarded as a true acetylcholinesterase (AChE). Pea aphids have been shown to contain cholinesterases which split acetylcholine and benzoylcholine but not acetyl-β-methylcholine (33).

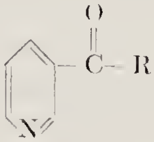
That the primary cause of organic phosphate poisoning in insects is due to the inhibition of the nerve acetylcholine-cholinesterase system has been strengthened by the proof that acetylcholine is present in the head of the blowfly (88), and common housefly (42). The presence of acetylcholine and two other unidentified choline esters has been demonstrated in the honey-bee head (13). Furthermore, it has been shown with honey-bee that the degree of organic phosphate poisoning is directly related to the brain AChE content (102). Numerous investigations have shown that good correlation exists between *in vitro* anticholinesterase activity and insect toxicity (56,40,130,102, 107,85).

The role of esterases other than cholinesterase in insect poisoning must also be regarded as of secondary or even primary importance in some cases. The extract of whole *Tribolium castaneum* Hbst. and *Tenebrio molitor* L., which hydrolyzed ethyl butyrate and *o*-nitrophenyl acetate, but not acetylcholine, is strongly inhibited by TEPP and *O,O*-diethyl *O-p*-nitrophenyl phosphorothionate (parathion), and some correlation was shown between toxicity and esterase activity. Known inhibitors of AChE such as prostigmine, carbamylcholine, and eserine were not toxic when injected into the locust, *Locusta migratoria migratoriodes*, whereas TEPP was found to be strongly toxic (73,74). The inhibition of enzymes hydrolyzing other esters may be the primary factor in poisoning in various developmental stages before the nervous system is fully developed.

The active center of the AChE surface is regarded as consisting of two primary regions, (*a*) a negatively charged site, which facilitates enzymic activity by attracting, binding, and orienting cationic substrates by virtue of coulombic attraction and van der Waals dispersion forces and (*b*) an "esteratic" site which is responsible for hydrolytic activity (143).

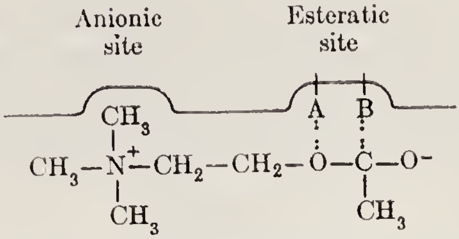
The presence of a negatively charged site is illustrated by the AChE inhibitory strengths of some ammonium ions. The following are values required to produce 50 per cent inhibition ($I_{50}$) by purified AChE of *Electrophorus electricus* in terms of molar concentration: $(CH_3)_4N^+$, $1.8 \times 10^{-2}$; $(CH_3)_3N^+H$, $1.5 \times 10^{-2}$; $(CH_3)_2N^+H_2$, $1.2 \times 10^{-1}$; $(CH_3)_3N^+CH_2CH_2OH$, $5.0 \times 10^{-3}$; $(CH_3)_2{}^+NHCH_2CH_2OH$, $5.0 \times 10^{-3}$; $CH_3{}^+NH_2CH_2CH_2OH$, $7.0 \times 10^{-2}$; ${}^+NH_3CH_2CH_2OH$, $2.8 \times 10^{-1}$; $(CH_3CH_2)_4N^+$, $5.0 \times 10^{-3}$; $(CH_3)_2{}^+NHCH_2CH_3$, $4.0 \times 10^{-3}$; and $(CH_3)_2CHCH_2CH_2OH$, $1.5 \times 10^{-1}$. It is apparent from these figures that positively charged ammonium salts are moderately attracted to the negatively charged site on the enzyme surface and that the degree of attraction increases in the order primary < secondary < tertiary = quaternary ammonium ion. On the average, there is a 6.7-fold increase in inhibition for each methyl group added to the nitrogen atom and this difference can be accounted for on the basis of van der Waals dispersion forces between the substrate and a hydrocarbon moiety of the enzyme. Dimethylaminoethanol ammonium ion is about 30 times as effective as its uncharged analogue isoamyl alcohol and this difference is attributed to electrostatic attraction of the ion to the negatively charged site.

The interpretation of pH dependency of enzyme activity suggests the presence of both electrophilic and nucleophilic groups at the esteratic site (143,145,146). In a series of nicotinic acid derivatives of the structure below the inhibitory strength was in the order R = OH < $NH_2$ < $CH_3$ = $N(C_2H_5)_2$ < $OC_2H_5$. This is to some degree the order of

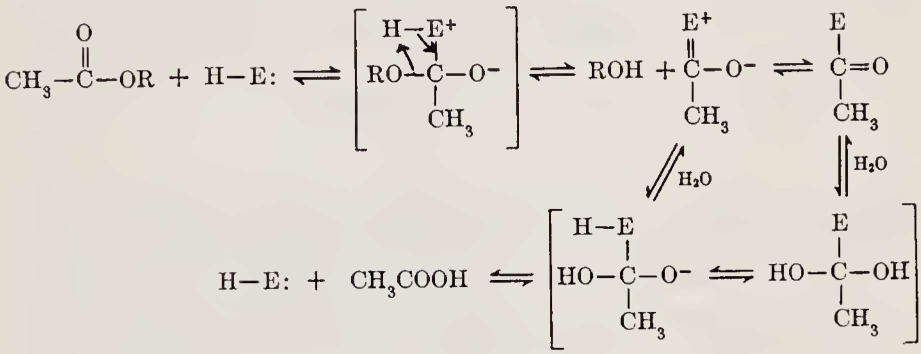


electron-withdrawing capacity of the R moiety and, therefore, a measure of the electron density around the carbonyl carbon. This interpretation, however, does not consider the effect of the —COR group on the nitrogen atom in the pyridine nucleus, since variations in R would change the basicity of the nitrogen and alter the attraction of the molecule to the negatively charged site of the enzyme. If this is disregarded, then it would appear that the nicotinic acid derivative is bonded in some manner by a nucleophilic attack of the basic group in the esteratic site on the carbonyl carbon atom. The acid group does not contribute to the formation of enzyme complexes (144) but is involved only in the hydrolytic process in which it functions in conjunction with the basic group to make a combined acid-base attack upon the substrate portion of the enzyme-substrate complex, resulting in the elimination of alcohol and the formation of an acetyl enzyme.

A hypothetical picture of the interaction between the two primary sites of the AChE molecule and acetylcholine molecule is given:

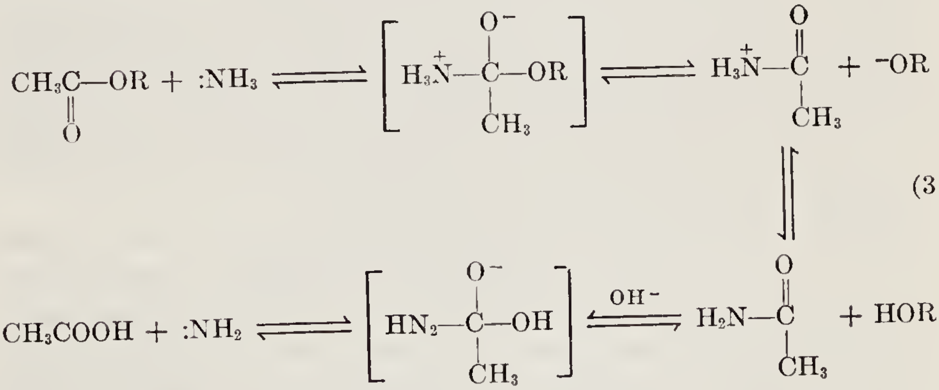


where A and B are the acid and basic groups. The mechanism of reaction involving the esteratic site may be depicted as follows:



(2)

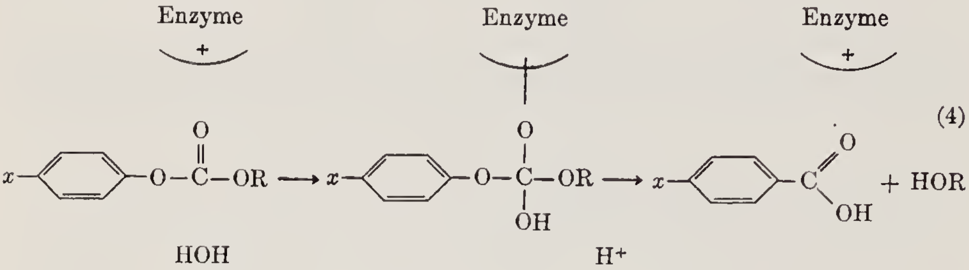
This is analogous to the mechanism proposed for the formation of amides from esters and ammonia and subsequent hydrolysis by hydroxide ion (75):



(3)

A mechanism similar to (2) above has also been proposed for the hydrolysis of substituted phenyl acetates by wheat germ lipase (65) since it was found that in a series of *ortho*-, *meta*-, and *para*-substituted phenyl acetates the rate of hydrolysis was parallel to the withdrawal of electrons from the acetoxy group, creating an electron deficiency at the carboxyl carbon atom.

A mechanism which differs from that proposed above in that the enzyme is first coordinated with the carboxyl oxygen atom as depicted below has been postulated from studies on the hydrolysis of substituted benzoyl cholines by horse serum cholinesteraee (122).

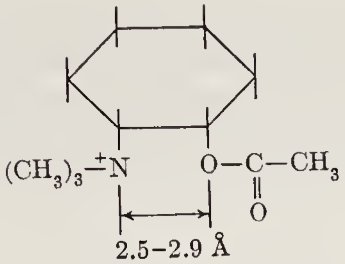


(4)

This mechanism is in agreement with that proposed for the inhibition of fly-brain AChE by phenyl *N*-methylcarbamates from studies in which a series of *meta*- and *para*-substituted phenyl *N*-methylcarbamates were examined and the order of increasing inhibitory activity was found to correspond to the order of increasing electron-releasing capacities of the substituents, thereby creating a greater electron density around the oxygen atom and facilitating the formation of a bond between the enzyme and the carbamate (85).

It should be pointed out here that the two mechanisms differ only in that the first (equation (2)) may be considered as a base-acid catalyzed hydrolysis of the substrate and the second (equation (4)) as an acid-base catalyzed hydrolysis. The difference is simply a matter of order but both should be considered to explain the inhibition of cholinesterase by different types of inhibitors.

Relatively little work is described concerning the precise distance of separation between the two primary sites in view of the flexibility of the two carbon chain in acetylcholine and the lack of knowledge of the particular molecular geometry assumed on its absorption at the catalytic surface. The higher rate of hydrolysis of *d,l-cis*-2-dimethylaminocyclohexyl acetate methiodide over the *trans*-acetate by AChE and the greater inhibitory activity of the *d,l-cis*-dimethylaminocyclohexanol methiodides over the *trans*-cyclohexanol would indicate that the distance between the nitrogen and oxygen atoms of the *cis* derivatives (2.5–2.9Å) is the nearest approximation to the distance between sites (27):



The *d,l-cis*-dimethylaminocyclohexanol methiodide is roughly twice as inhibitory as the *trans*-cyclohexanol and the *cis*-acetate is hydrolyzed about 8 per cent faster than the corresponding *trans*-acetate.

Although the esters of choline are considered as the natural substrates for the cholinesterases, these enzyme can also hydrolyze esters of other types. The ability of human erythrocyte and cobra venom AChE and human plasma ChE to hydrolyze a series of *p*-substituted phenyl acetates has been demonstrated (111). Both AChE from human erythrocyte and ChE from human plasma hydrolyze a variety of aliphatic esters and do so most rapidly with those which most closely approach the choline configuration, but in general, at a rate less than the corresponding choline ester (1). Breis from fly, bee, and cockroach heads also hydrolyze a wide variety of aliphatic esters. With fly brei, hydrolysis is at a maximum with 3,3-dimethylbutyl acetate, the carbon analogue of acetylcholine, and with bee brei hydrolysis is at a maximum with isoamyl acetate (106). These facts point out that high enzyme-substrate affinity can occur with neutral substrates provided the overall configuration of the molecule is similar to that of acetylcholine. This is discussed further in a later section in relation to the mechanism of action of some inhibitors.

## III. The Mechanism of Inhibition of Esterases by Reversible and Irreversible Inhibition

The inhibitors of esterases may be divided into two distinct types, the reversible or competitive inhibitors and the irreversible inhibitors. This classification is based partly on the regeneration rate of the inhibited enzyme; the alkyl carbamates and quaternary ammonium compounds are examples of reversible inhibitors, compared to the organic phosphates which are considered irreversible inhibitors.

### A. REVERSIBLE INHIBITION

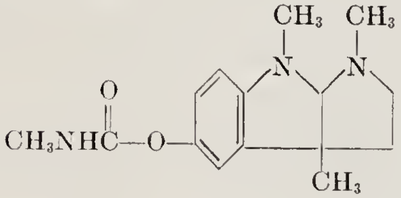
The inhibitory power of reversible inhibitors is presumably due to a strong affinity for the enzyme occasioned by their similarity in shape to the substrate molecules, combined with a stability not possessed by carboxylic esters when absorbed on to the active centers. Qualitatively the difference in substrate and inhibitor may be pictured as follows where E = enzyme, S = substrate, I = inhibitor and X,Y, X′,Y′ products:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + X + Y \qquad (5)$$

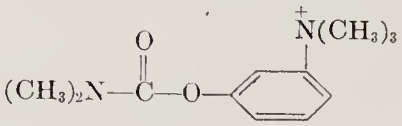
$$E + I \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} EI \underset{k_4'}{\overset{k_3'}{\rightleftharpoons}} E + X' + Y' \qquad (6)$$

In the substrate hydrolysis the enzyme substrate complex ES is rapidly degraded to the original enzyme E and hydrolyzed products X and Y; in enzyme inhibition the enzyme-inhibitor complex EI is relatively stable and regeneration to E and I, or if possible, hydrolysis to products X′ and Y′ occurs very slowly. S and I both compete for the same enzyme E and the degree of inhibition is determined by the rate constants shown in equations (5) and (6). The kinetics of these two competing reactions have been fully worked out and the values for $k_1/k_2$ and $k_1'/k_2'$ can be determined experimentally (95).

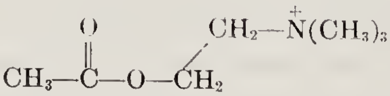
A wide variety of compounds inhibit cholinesterase by this mechanism. Physostigmine (I) and prostigmine (II) are classical examples of reversible inhibitors which structually closely resemble acetylcholine (III).



(I)



(II)



(III)

Quaternary ammonium compounds (143), various amino acids (146), methylene blue (12), and a wide variety of alkyl carbamates (85) inhibit cholinesterase by competing for either one or both of the active sites on the enzyme surface.

Studies on the mechanism of inhibition by these competitive inhibitors have aided us in our understanding of the nature of the active sites. It has been demonstrated in a series of *meta*- and *para*-substituted phenyl *N*-methylcarbamates that the degree of inhibition of acetylcholine hydrolysis by fly-brain AChE by these compounds is directly related to the electron releasing capacity of the substituent (85). In Fig. 1 is plotted $-\log K_i$ ($K_i$ is the inhibition constant $= ki'/k_2'$ in equation 6) versus Hammett's sigma values for various substituents in the *meta* and *para* positions. Hammett's sigma values are a measure of the electron attracting or releasing capacities of the substituents (66); electron-releasing groups have negative values and electron-attracting groups have positive values. Fig. 1 shows that compounds containing substituents which increase the electron density around the carbamate moiety are most potent as inhibitors

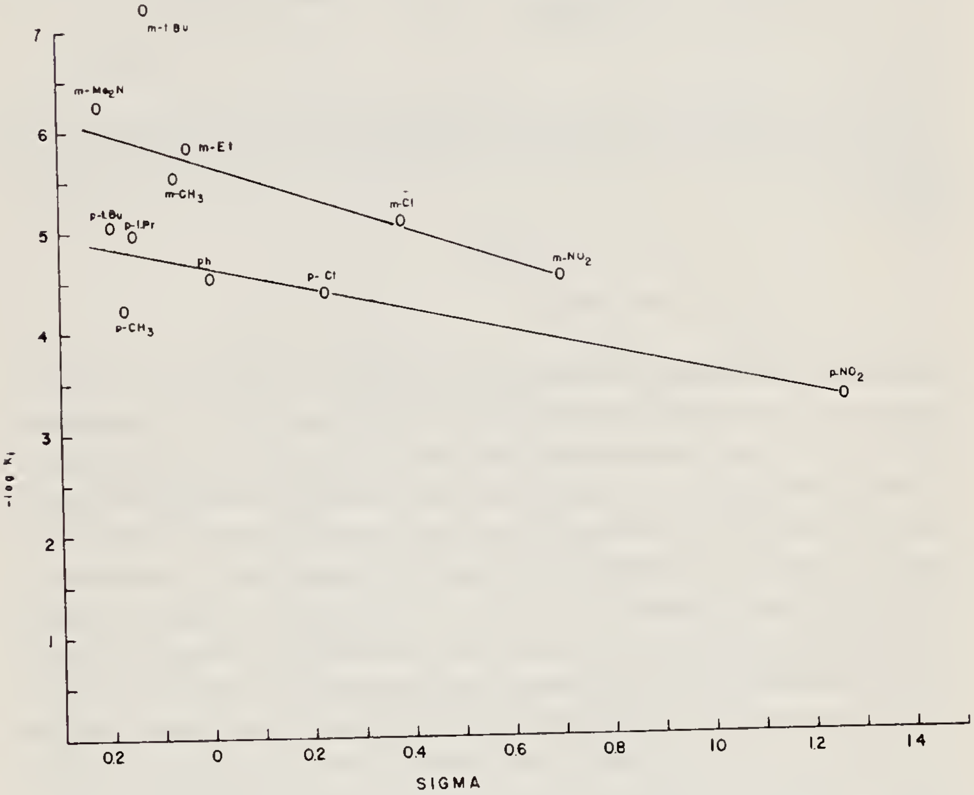
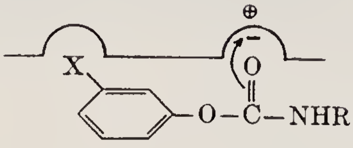
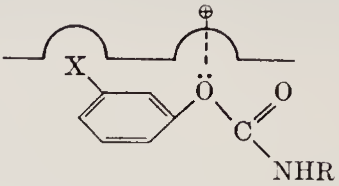


Fig. 1. Plot of $-\log K_i$ vs. Hammett's sigma values for substituted phenyl *N*-methylcarbamates (85).

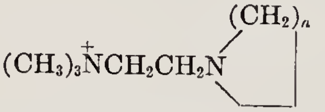
and suggests that the inhibition is attributed in part to strong binding between a locus of high electron density in the carbamate linkage and the esteratic site. A typical inhibitor-enzyme complex may be designated as:



or



Further evidence supporting an inhibitor-enzyme complex of this nature is obtained from the observation that compounds of the general type



(IV) $n = 2$
(V) $n = 3$

are potent competitive inhibitors of the catalyzed hydrolysis of acetylcholine by AChE from *Electrophorus electricus* (54). Compounds (IV) and (V) are, respectively, 2.7, and 3.8 times more effective in covering catalytic sites than the most powerful natural inhibitor, physostigmine (I). The relative efficiency of IV and V, differing in essential structure chiefly at the angular disposition of groups about the ring nitrogen, seems to be in line with the relative order of basicities of the secondary ring bases, i.e., $K_b$ values in water at 25°C. for pyrrolidine and piperidine are, respectively, 1.3 and $1.6 \times 10^{-3}$. Greater basicity at the ring nitrogen function infers greater electron localization on the nitrogen atom resulting in increasing inhibiting powers. In this regard, cholyl chloride, $(CH_3)_3\overset{+}{N}CH_2CH_2Cl$, which contains a primary chloro function serving as a region of high electron density is more effective as an inhibitor than prostigmine (II) (55).

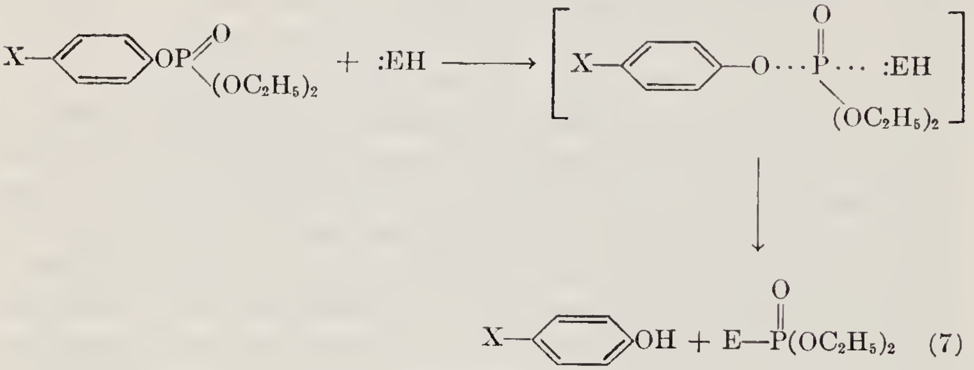
## B. IRREVERSIBLE INHIBITION

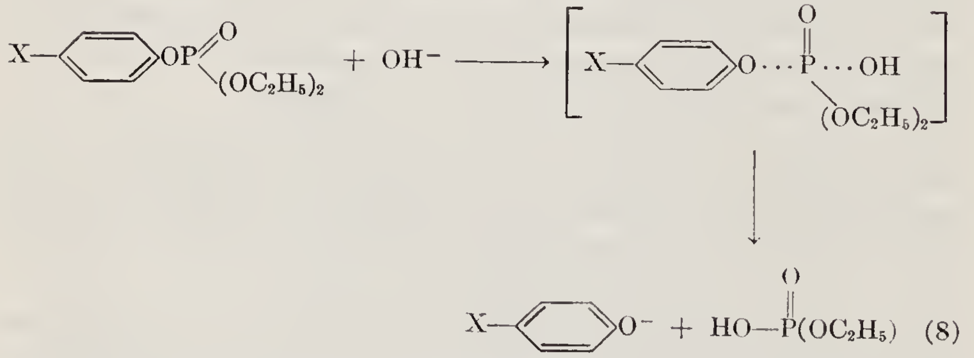
The recovery rate of ChE inhibited by the organophosphorus compounds is slow compared to the recovery rate of ChE inhibited by competitive inhibitors. For example, ChE inhibited by a carbamate returns to its normal activity in a matter of a few hours after removal of the excess carbamate; ChE inhibited by an organic phosphate, however, returns to its normal activity after several days. The difference in recovery rate can be explained on the basis that the inhibition of an enzyme by an organic phosphate is not due to competitive absorption on the enzyme surface, but is a result of an irreversible chemical reaction in which the enzyme is phosphorylated.

The reaction mechanisms between organophosphorus compounds and various esterases have been studied in much detail. That actual chemical reaction occurs between phosphate and enzyme has been demonstrated with several different phosphorus compounds and enzymes. Both proteinase and esterase activities of pure, crystalline $\alpha$, $\beta$, and $\gamma$ chymotrypsin are inhibited by DFP (80,78,53) by equimolar reaction of the phosphate with the enzyme in which one di*iso*propylphosphoryl moiety is introduced into the enzyme. This stoichiometry between enzyme and phosphate was shown by phosphorus analysis of the inactive enzyme, by the use of $P^{32}$-labeled DFP (81), and the liberation of an equivalent amount of hydrofluoric acid (82). Analysis of crystalline $\alpha$-chymotrypsin inactivated by TEPP, diphenyl phosphorochloridate, tetra*iso*propyl pyrophosphate, diethyl phosphorothionofluoridate, para-oxon, and tetrapropyl dithionopyrophosphate showed that in each case approximately one molecule of phosphorus had been introduced per molecule of enzymes in spite of differences in reactivity of these compounds (79). Para-oxon and $\alpha$-chymotrypsin react with the liberation of an equivalent amount of *p*-nitrophenol and the rate of liberation is equal and exactly corresponds to the rates of inhibition of the proteinase and esterase activities (68). Furthermore, the kinetics of liberation of *p*-nitrophenol are explained on the basis of a bimolecular reaction between enzyme and inhibitor.

The reaction between various organophosphorus compounds and the cholinesterase enzymes have also been investigated. AChE from *Electrophorus electricus* and $P^{32}$-labeled DFP have been shown to react with the introduction of phosphorus into the enzyme (110). This phosphorus is tightly bound to the enzyme and cannot be removed by prolonged dialysis or treatment with trichloroacetic acid at room temperature. Cholinesterase prepared from horse serum and $P^{32}$-labeled DFP also react to form a phosphorus-containing enzyme which retained all its radioactivity even after prolonged extraction with organic solvents (30a).

Although the stoichiometry of the reaction has not been determined because of the uncertainty in the molecular weights of the cholinesterases, equimolar reaction is inferred from kinetic studies (6,3). The kinetics of the inhibition of sheep erythrocytes AChE by several substituted phenyl diethyl phosphates (para-oxon analogues) have been examined and shown to be first order with respect to phosphate concentration and the overall reactions as bimolecular. The bimolecular rate constants for these reactions have been determined and in general they are parallel to the rate of aqueous hydrolysis of the phosphates. A linear relationship is obtained when the logarithm of the hydrolysis constant is plotted against the negative logarithm of the enzyme reaction constant as shown in Fig. 2, which indicates that the two processes of inhibition and aqueous hydrolysis are probably analogous and illustrated by the equations below where :EH is the enzyme.

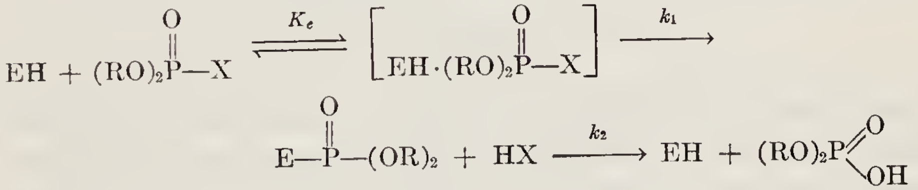

(7)


(8)

It is not proposed that these equations represent the actual mechanism of the reactions but they can be considered as possible mechanisms and are given merely to point out the similarity between enzyme reaction and aqueous hydrolysis. The linear relationship between hydrolysis and enzyme reaction further points out that the rate of enzyme inhibition is, in part, a function of the lability of the P—O bond, and this is discussed in a later section.

Knowledge of the exact nature of the functional groups present in the esteratic site in the cholinesterase molecule which react with the organophosphorus compound is still somewhat diffuse. DFP, TEPP, and di*iso*propyl phosphorochloridate have been shown to react with certain amino acids and phenols to give definite compounds (139,77). The kinetics of the reaction between *iso*propyl methylphosphonofluoridate (Sarin) with catechols have been studied and the overall reactions have been shown to be bimolecular as in enzyme inhibition (51). DFP has also been shown to form an unstable complex with imidazole which has been suggested as a functional group in the ChE molecule and it has been proposed that phosphorylation of the enzyme occurs through the imidazole group (138,145).

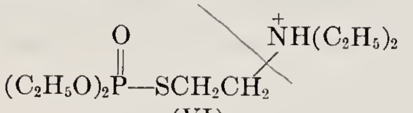
A generalized mechanism for the inhibition of esterases by the organophosphorus compounds has been proposed which may be depicted as follows (68,3), where R is any short chain alkyl group and

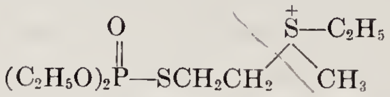


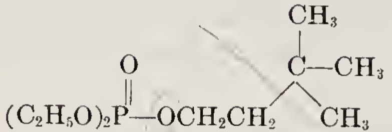
$$EH + (RO)_2\overset{\overset{O}{\|}}{P}-X \overset{K_e}{\rightleftharpoons} \left[EH\cdot(RO)_2\overset{\overset{O}{\|}}{P}-X\right] \xrightarrow{k_1} E-\overset{\overset{O}{\|}}{P}-(OR)_2 + HX \xrightarrow{k_2} EH + (RO)_2P\begin{smallmatrix}\nearrow O\\ \searrow OH\end{smallmatrix} \qquad (9)$$

X is any displaceable group (e.g., halogen, alkoxy, or aryloxy). The first step in the inhibition process is the formation of the enzyme-inhibitor complex which is determined by the equilibrium constant $K_e$. In general, this complex should not be regarded as being similar in nature to the complexes formed by the reversible inhibitors discussed in the previous section, but as the transition state leading to the subsequent formation of the phosphorylated enzyme determined by the rate constant $k_1$. The interpretation of the enzyme-inhibitor complex as a transition state type intermediate is supported by the fact that reversible inhibitors such as procaine and eserine prevent the reaction between AChE from a variety of sources and organic phosphates such as TEPP and Tabun from taking place (20,11). Were the enzyme-organic phosphate complex similar to the reversible inhibitor-enzyme complex, then equilibrium should be established and some degree of phosphorylation would be expected. The protective action of a reversible inhibitor can be regarded as resulting from strong adsorption of the inhibitor on the active sites, thus blocking the reaction between the phosphate and enzyme.

There are, however, a number of organic phosphates in which the reactivity of the P—O bond alone does not account for their extremely high inhibitory activity. Such compounds as *O,O*-diethyl *S*-2-diethylaminoethyl phosphorothiolate oxalate (VI), $I_{50}$ $3 \times 10^{-9}M$ (64), *O,O*-diethyl *S*-2-ethylmercaptoethyl phosphorothiolate methosulfate (VII), $I_{50}$ $3.3 \times 10^{-8}M$ (59), and diethyl 3,3-dimethylbutyl phosphate (VIII), $I_{50}$ $3 \times 10^{-7}M$ (57) are typical examples of a group of organophosphorus compounds which structurally closely resemble acetylcholine, the cationic forms which are shown:


(VI)

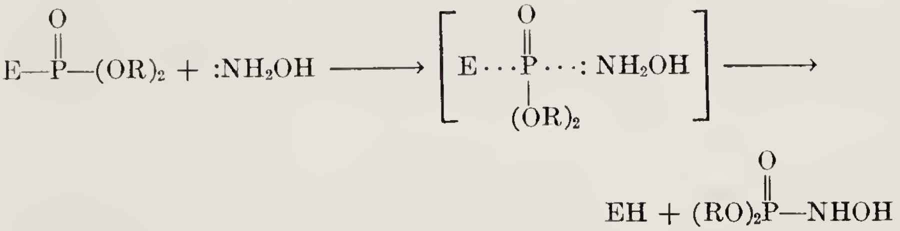

(VII)


(VIII)

Because of their structural similarity to acetylcholine these compounds would be expected to be adsorbed on the enzyme surface in the same manner in which acetylcholine is attached to the enzyme, a process which brings the inhibitor in close proximity to the active sites, thereby facilitating the phosphorylation reaction. The high inhibiting strength of these compounds, therefore, depends upon favorable values of both $K_e$ and $k_1$ in equation (9).

The regeneration of enzyme depends upon the hydrolysis of the phosphorylated enzyme as determined by the rate constant $k_2$ in equation (9). The rate of regeneration appears to be a function of the group R in the organic phosphate molecule and is in the order R = methyl > ethyl > *iso*propyl. The enzyme activities of rabbit erythrocytes ChE inhibited by *O,O*-dimethyl *O*-*p*-nitrophenyl phosphate, *O,O*-dimethyl phosphorofluoridate, tetramethyl pyrophosphate, and *O,O*-dimethyl *O*-*p*-nitrophenyl phosphorothiolate were found to return at the same rate (7). The half-life of the inhibited enzyme at 37°C. and pH 7.8 is about 85–90 minutes. With the ethyl analogues of the same compounds, the return of enzyme activity is also the same for all four compounds with a half-life of about 150 minutes. In comparison, the regeneration of ChE and AChE inhibited by organophosphorus compounds containing di*iso*propoxy and di*iso*propylamino moieties is so slow that the inhibition may be considered as essentially irreversible (44).

The mechanism of regeneration of enzyme probably involves enzymatic and nonenzymatic hydrolysis of phosphorylated enzyme. Evidence for nonenzymatic hydrolysis is apparent in the large differences in the regeneration rate of inhibited enzymes containing different phosphate moieties and is readily explained on the differences in stability of the phosphorylated enzyme to aqueous hydrolysis as predicted from electronic and steric considerations. The order of rate of solvolysis in ethanol of dimethyl, diethyl, and diisopropyl phosphorochloridates is dimethyl > diethyl > di*iso*propyl which corresponds in order to the regeneration rate of enzymes inhibited by these groups (46). In this connection, certain nucleophilic reagents greatly enhance the reactivation process, presumably by a direct displacement of the enzyme from the phosphorus atom (148) as illustrated below with hydroxylamine.



$$E-\overset{O}{\overset{\|}{P}}-(OR)_2 + :NH_2OH \longrightarrow \left[ E\cdots\underset{(OR)_2}{\underset{|}{\overset{O}{\overset{\|}{P}}}}\cdots:NH_2OH \right] \longrightarrow EH + (RO)_2\overset{O}{\overset{\|}{P}}-NHOH \qquad (10)$$

Since AChE has been shown to contain an anionic site it is expected that a good reactivator could be produced by combining in the same molecule a strong nucleophilic group and a positive-charged quaternary ammonium group. Nicotinhydroxamic acid methiodide and $\alpha$-pyridylaldoxime methiodide possess these qualities and are extremely efficient reactivators of TEPP and DFP inhibited AChE and the latter shows promise as a therapeutic agent in phosphate poisoning (147).

Enzymatic hydrolysis of the phosphorylated enzyme must also be regarded in the reactivation process in view of the presence of enzymes which catalyze the hydrolysis of organophosphorus compounds. Enzymes found in different tissues of both mammals and insects and capable of hydrolyzing a variety of organophosphorus esters have been described in a previous section.

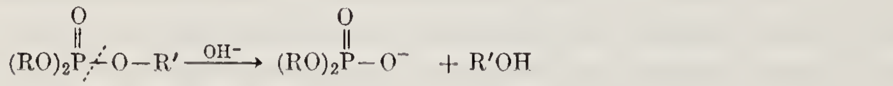
## IV. Relationship Between Structure and Activity

It has been firmly established in the preceding section that enzyme inhibition by an organophosphorus compound is brought about by a chemical reaction between the phosphorus compound and the enzyme with the formation of a covalent type bond. The velocity of this reaction which determines the degree of inhibition depends largely on the reactivity of the phosphorus compound. Certain relationships exist, e.g., the hydrolysis or solvolysis rates of these inhibitors, which can be correlated with the inhibition velocity.

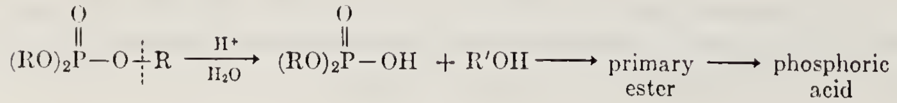
### A. REACTION MECHANISMS

Phosphorus atoms in compounds which inactivate esterases exist in the +5 oxidation state. Included in this family of inhibitors are phosphates, phosphonates, pyrophosphates, phosphorothiolates, and various phosphoroamidates. The reaction between enzyme and the organophosphorus compound appears to involve the cleavage of the bond directly connected to the phosphorus atom as illustrated in equation (7) with para-oxon. Tertiary phosphate esters can be hydrolyzed readily to the secondary esters and the bond which is broken depends upon the structure of the compound and hydrolytic conditions.

Studies of the mechanism of hydrolysis in $O^{18}$-rich water of a tertiary ester $(RO)_2\overset{\overset{O}{\|}}{P}—O—R'$, show that in alkaline solution the usually expected displacement of the OR′ group occurs (30). In acidic or "water" hydrolysis a rupture of the O—R′ link takes place, at least in the first step. Usually the secondary esters are quite stable to alkaline hydrolysis, and further attack occurs only under fairly drastic conditions. In acids the hydrolysis of the secondary esters proceeds rather rapidly, but generally at a slower pace than shown in the hydrolysis of tertiary esters (26,29). The hydrolysis of simple organic phosphates in acids and in bases, therefore, can be summarized as follows:

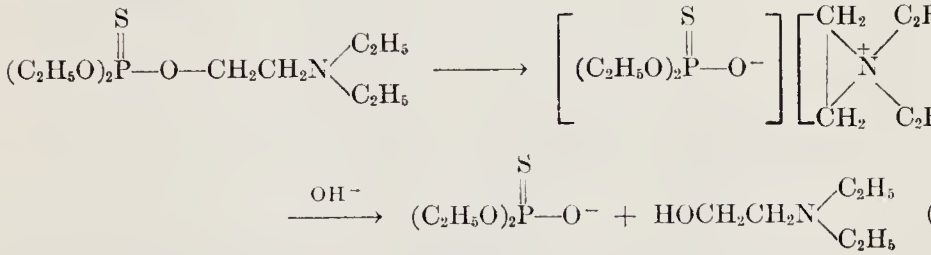


$$(RO)_2\overset{\overset{O}{\|}}{P}\,\vdots\,O-R' \xrightarrow{OH^-} (RO)_2\overset{\overset{O}{\|}}{P}-O^- + R'OH \qquad (11)$$



$$(RO)_2\overset{\overset{O}{\|}}{P}-O\,\vdots\,R \xrightarrow[H_2O]{H^+} (RO)_2\overset{\overset{O}{\|}}{P}-OH + R'OH \longrightarrow \text{primary ester} \longrightarrow \text{phosphoric acid} \qquad (12)$$

A number of organophosphorus compounds, however, do not follow this general rule of acidic and basic hydrolytic cleavage. Cleavage of the P—O or R′—O bond depends upon the nature of the group R′. For example, malathion, *O,O*-dimethyl *S*-1,2-dicarboethoxyethyl phosphorodithioate, is split by strong alkali between the carbon and sulfur atoms giving *O,O*-dimethyl phosphorodithioic acid as one of the products (23). *O,O*-Diethyl *O*-2-diethylaminoethyl phosphorothionate and phosphate are both cleaved in alkali between the diethylaminoethyl groups and the oxygen atom (61) and kinetic studies show that the hydrolysis probably occurs through the formation of an immonium ion.


(13)

The isomer, *O,O*-diethyl *S*-2-diethylaminoethyl phosphorothiolate, a powerful inhibitor of fly-brain AChE and which does not form an immonium ion, however, is split between the phosphorus and sulfur atoms by alkali. The hydrolysis of phosphorus compounds, such as ethyl *N,N*-dimethylphosphoroamidocyanidate (tabun), which contain different groups attached to the phosphorus atom proceeds by two paths depending upon the conditions (87). In acid the P—N bond between the phosphorus atom and dimethylamido nitrogen is split and in base the cyanide group is displaced. The enzymatic hydrolysis of tabun by rabbit and horse plasma is similar to basic hydrolysis and only cyanide ion is liberated (19).

It appears that the mechanism of reaction between enzyme and phosphorus compound and the basic hydrolysis of the phosphorus compound are similar. It is expected, therefore, that to a degree the most potent inhibitors of the cholinesterases are those which are most easily hydrolyzed under basic conditions (Fig. 2). The kinetics of the basic hydrolysis of the substituted phenyl diethyl phosphates listed in Fig. 2 were shown to be bimolecular and their rates paralleled the rates of reaction with sheep erythrocyte AChE.

The mechanism of hydrolysis of the secondary halophosphates to the acid has been studied to a greater extent than the hydrolysis of tertiary phosphates, largely because of relatively simple methods of analysis. The hydrolysis of di*iso*propyl phosphorofluoridate (DFP) is catalyzed by hydrogen ion (140). The rate of liberation of fluoride ion was first order and increased with increasing hydrogen ion concentration. The first order rate constant ($k$, hr.$^{-1}$) at 25°C. increased from 0.006 at pH 7 to 0.225 at pH 1.3. In alkaline solution DFP hydrolyzes much more rapidly; at equivalent concentrations the liberation of fluoride ion is complete in about 15 min. at 25°C. The diethyl analogue, in comparison, is hydrolyzed about 25 times more rapidly in water than DFP (96).

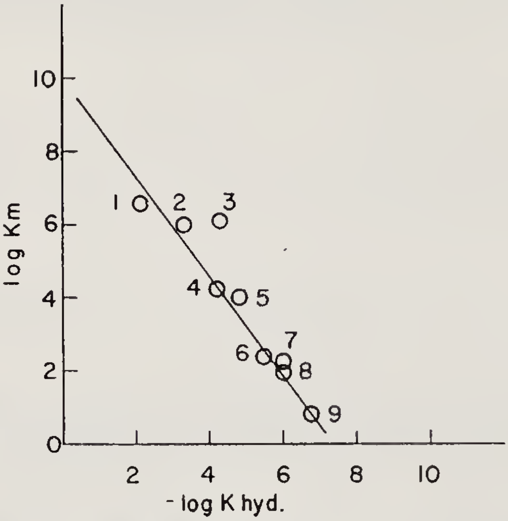


Fig. 2. Relation between hydrolysis constant $K_{hyd.}$ and bimolecular rate constant $K_m$ for reaction with ChE for organic phosphate esters (6). 1, tetraethyl pyrophosphate; 2, *S*-phenyl parathion; 8, parathion; and the following substituted phenyl diethyl phosphates; 3, *p*-$NO_2$; 4, *o*-$NO_2$; 5, *m*-$NO_2$; 6, *o*-Cl; 7, *p*-Cl; and 9, unsubstituted (from Metcalf (101)).

It appears from studies on the solvolysis and reactions of various phosphorochloridates with amines and anionic reagents that the chloride ion is liberated by simple displacement at the phosphorus atom (46–49). The first order rate constants for the solvolysis of phosphorochloridates, $Cl—PO(OR)_2$, in dry and in 80% aqueous ethanol are given in Table I. The data in Table I show that the solvolysis of the halide occurs more rapidly in 80% than in 100% ethanol. More detailed studies on the solvolysis of di*iso*propyl phosphorochloridate

TABLE I

First Order Rate Constants for the Solvolysis of Phosphorochloridates, Cl—PO(OR)$_2$, in Dry Ethanol and in 80% Ethanol

| | | $K \times 10^4$ (sec.$^{-1}$) | |
|---|---|---|---|
| R | Temp., ° C. | 100% EtOH | 80% EtOH |
| $CH_3$ | 39.6 | 9.5 ± 0.6 | 76 ± 2 |
| $C_2H_5$ | 39.6 | 5.1 ± 0.4 | |
| | 25.14 | 1.7 ± 0.2 | 13.7 ± 0.5 |
| $i$-$C_3H_7$ | 39.6 | 1.78 ± 0.03 | 9.5 ± 0.5 |
| | 25.2 | 0.61 ± 0.02 | 3.0 ± 0.1 |
| $CH_2C_6H_5$ | 39.6 | 11 ± 1 | 64 ± 1 |
| | 25.2 | 3.5 ± 0.3 | |
| $C_6H_5$ | 39.6 | 8.5 ± 0.5 | |
| | 25.2 | 3.2 ± 0.2 | 63 ± 1 |

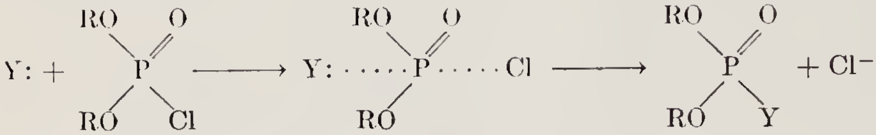
From (46) I. Dostrovsky and M. Halmann, *J. Chem. Soc.*, *1953*, 502.

showed that the change of solvent from less aqueous to a more aqueous medium accelerates the solvolytic reactions. However, the rate of solvolysis of di*iso*propyl phosphorochloridate increases greatly on passing from formic acid to aqueous ethanol of equal ionizing capacity. In solvolysis which is partly or wholly bimolecular, a change from relatively strongly nucleophilic aqueous ethanol to the very weakly nucleophilic formic acid will lead to a great reduction in rate. The differences in rates of solvolysis of di*iso*propyl phosphorochloridate in the two solvents points to a bimolecular mechanism for the reaction.

The kinetics of the reaction between phosphorochloridates and anionic reagents were also investigated (47) and found to be simple bimolecular substitution reactions. The order of reactivity of some of the reagents studied can be listed as $F^- > CH_3CH_2O^- > C_6H_5O^- > CH_3CH_2OH > C_6H_5S^- > CH_3COO^-$. The effects of changes of the alkyl group R in $(RO)_2P(O)Cl$ in the reaction with fluoride and ethoxy ions were determined and found to be in the same order as in solvolysis, i.e., $CH_3 > C_2H_5 > i\text{-}C_3H_7$ (see Table I).

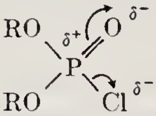
The reactions of dimethyl, diethyl, and di*iso*propyl phosphorochloridate with aliphatic primary, secondary, and tertiary amines are also of second order, the rate decreasing in going from dimethyl to the di*iso*propyl ester (48). It was found that secondary amines react much more slowly than the corresponding primary amines with di*iso*propyl phosphorochloridate. Furthermore, branching in the α position greatly slows down the rate, e.g., *n*-butylamine reacts faster than *sec*-butylamine which reacts faster than *tert*-butylamine, indicating the operation of steric factors. On the other hand, aniline reacted slower than all aliphatic amines studied, which is in keeping with its much weaker basicity.

The kinetic analysis of the reactions described above leads to the conclusion that the solvolysis and reactions with strong nucleophilic reagents proceed by the same mechanism. The reaction may be written as follows:


(14)

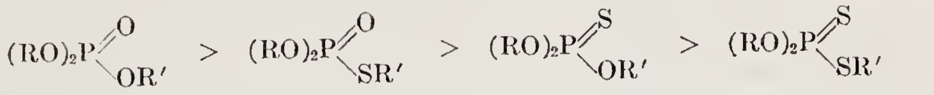
where Y: is any nucleophilic reagent. In enzyme inhibition, Y: may be pictured as a nucleophilic group attached to the esteratic site.

The effect of structural changes in the phosphorochloridates may be understood by considering the possible interaction of the groups RO with P═O oxygen atom in the compound. On account of the electronegativity difference between oxygen and phosphorus (2.1 for P and 3.5 for 0), and between chlorine and phosphorus, the phosphorus atom will carry a small positive charge and be the electrophilic center of the molecule (123). Any electron release from the group RO will



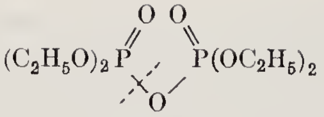
weaken this center, which may be caused by a positive inductive or by a positive electromeric effect in the group R (144). Using the well established principles governing these effects, the groups RO may be arranged in order of their electron release to the phosphorus atom, $CH_3O < CH_3CH_2O < (CH_3)_2CHO$, which is in agreement with the rates of solvolysis shown in Table I. The high rates of solvolysis of the dibenzyl and diphenyl phosphorochloridates is probably a result of high electron withdrawal by the phenyl groups.

It is clear from these considerations that the reactivity of the organophosphorus compound depends upon the electrophilic character of the phosphorus atom which is a function of the groups attached to it. A general order of electron-releasing character of groups most commonly attached to the phosphorus atoms in organophosphorus insecticides can be arranged as follows: $F < OR < SR < NR_2$, where R is a simple alkyl group. This scale tells us that F is better in inducing electrophilic character to the phosphorus atom than OR, which in turn is better than SR, etc. The electronegativity difference between phosphorus and the thiono sulfur is small (2.1 for P and 2.5 for S) and therefore the phosphorus atom in phosphorothionates and phosphorodithioates is not expected to carry as large a positive charge as the phosphorus atom in phosphates. In view of these considerations the sulfur analogues of the phosphates, e.g., phosphorodithioates, phosphorothionates, and phosphorothiolates are expected to be weaker inhibitors than the phosphates and this is exemplified in numerous examples. Pure parathion, the sulfur analogue of paraoxon, shows little or no inhibition of cholinesterase (45,104). *O,O*-Diethyl *O*-ethyl-2-mercaptoethyl phosphorothionate (thiono isomer of Systox) is about 1000 times less effective as an inhibitor of fly-brain AChE than the corresponding thiol isomer, which in turn is 100 times less effective than the corresponding phosphate (60). Several phosphorodithioates which are currently being evaluated as insecticides such as *O,O*-dimethyl *S*-2-methyl-4-ketobenzotriazine phosphorodithioate (Bayer 17147) and *O,O*-diethyl *S*-ethylmercaptomethyl phosphorodithioate (Thimet) do not inhibit AChE when absolutely pure (105). Based on the effects of the various groups on the electrophilic character of the phosphorus atom, the order of inhibitory activity of an organic phosphate and its sulfur analogues may be arranged as follows:

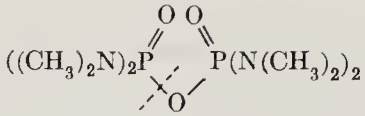


$$(RO)_2P(=O)OR' > (RO)_2P(=O)SR' > (RO)_2P(=S)OR' > (RO)_2P(=S)SR'$$

According to the position of $R_2N$ in the above scale in which is arranged the order of electron release, organophosphorus compounds containing dialkylamino groups, in general, are expected to be rather poor inhibitors of cholinesterases. A good example of this is the very weak inhibiting activity of octamethylpyrophosphoramide (Schradan) and its analogues compared to TEPP which is one of the most powerful inhibitors known. The anhydride bond in TEPP shown below is very labile and is readily split by nucleophilic reagents (134).



TEPP

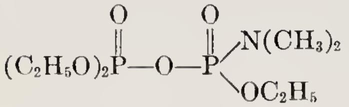
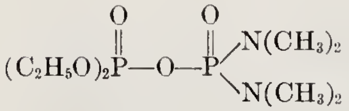
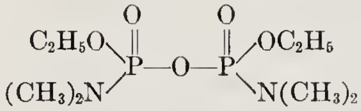
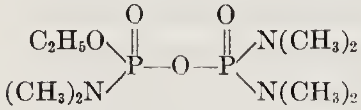


Schradan

In Schradan, however, the electrophilic center on the phosphorus atom is weakened by electrons from the dimethylamino groups, resulting in a P—O bond which is highly resistant to attack by nucleophilic reagents. The rate constant, $K_b$, for alkaline hydrolysis of Schradan is $6 \times 10^{-6}[OH^-]$ min.$^{-1}$ at 25°C.; its half-life in 1*N* alkali, therefore, calculates out to be about 70 days. In comparison the half-life of TEPP in water is 6.8 hours (134). The effect of the dialkylamino group on the reactivity of the P—O bond is further exemplified by examining the effect of progressive substitution of ethoxy groups in TEPP with dimethylamino groups. The values for the relative hydrolysis rates at pH 7.4, 37°C., and the relative effectiveness as inhibitors of chymotrypsin by TEPP, Schradan, and the various intermediates are given in Table II (34). It is apparent

TABLE II

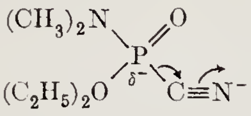
Relative Hydrolysis Rates and Effectiveness as Chymotrypsin Inhibitors of TEPP and Schradan Intermediates

| | Rel. hyd. rate | Rel. chymotrypsin inhibition |
|---|---|---|
| TEPP | 5.0 | 4.7 |
|  $(C_2H_5O)_2P(O)\text{—O—}P(O)(N(CH_3)_2)(OC_2H_5)$ | 2.9 | 3.5 |
|  $(C_2H_5O)_2P(O)\text{—O—}P(O)(N(CH_3)_2)_2$ | 2.8 | 2.8 |
|  $(C_2H_5O)((CH_3)_2N)P(O)\text{—O—}P(O)(OC_2H_5)(N(CH_3)_2)$ | 2.7 | 2.4 |
|  $(C_2H_5O)((CH_3)_2N)P(O)\text{—O—}P(O)(N(CH_3)_2)_2$ | 1.8 | 0.1 |
| Schradan | 0 | 0.1 |

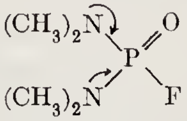
From J. Casida, T. Allen, and M. Stahmann (34).

from these data that progressive substitution lowers both the hydrolysis rates and inhibitory activities.

The stabilizing effect of dialkylamino groups to attack by nucleophilic reagents, however, can be overcome by attaching strong electron-attracting groups on the same phosphorus atom. Ethyl *N*,*N*-dimethylphosphoroamidocyanidate (tabun), a powerful AChE inhibitor, illustrates the influence of a group such as CN on the overall reactivity of the molecule:

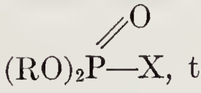


Tabun



Dimefox

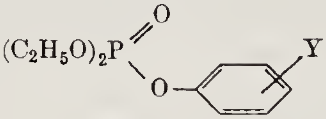
The CN group is capable of attracting electrons away from the phosphorus atom, inducing an electrophilic center in spite of the tendency of dimethylamino group to weaken this center. However, the effect of two dialkylamino groups cannot be overcome by electronegative groups, as illustrated by dimefox which is a weak anticholinesterase.

It is apparent from the discussions on the mechanism of inhibition of esterases by organophosphorus compounds that at least two requirements must be satisfied for high inhibition; firstly, rapid reaction between enzyme and phosphate, and secondly, reasonable stability of the phosphorylated enzyme. These criteria, therefore, require that there be present in the molecule a strong electron-attracting group which is easily displaced by enzyme. The resulting phosphorylated enzyme, in order to be stable to hydrolysis, must now possess groups attached to the phosphorus atom which are weakly electron attracting or even electron releasing. However, the reactivity of an organophosphorus compound is not solely a function of the electronic properties of the groups attached to the phosphorus atom, but depends also on their steric properties. In organophosphorus compounds of the type  $(RO)_2P(=O)—X$, the large drops in reactivity of the P—X bond as R proceeds from ethyl to propyl to butyl, etc., is not in line with the small differences in electron release by R, but also appears to be a function of the size of R. In tetraalkylpyrophosphates, when R becomes too large or highly branched, the hydrolysis rate will slow down due to simple physical interference by R (32,135), a phenomenon referred to as "steric inhibition." The selection of R is, therefore, limited to methyl, ethyl, *iso*propyl (or their equivalent in phosphoroamidates) for most rapid enzyme reaction.

Variations in the group X are apparently limitless. In view of the duality of the active site on the enzyme surface selection of X can be made on two fundamental premises: (*a*) a group X which will induce electrophilic character to the phosphorus atom, consequently, a highly reactive P—X bond and (*b*) a group X of such structure that the overall configuration of the organophosphorus molecule is similar to acetylcholine but also possessing a moderately reactive P—X bond.

Strongly electron-withdrawing groups include the halogens, cyanide, and an unlimited number of others which withdraw electrons either by inductive, mesomeric, or electromeric effects (75); the latter two are resonance effects.

Studies in which a series of diethyl substituted phenyl phosphates were examined for insect toxicity and inhibition of fly-brain AChE showed that remarkable parallelism existed between these properties and the reactivity of the P—O-phenyl bond as determined by hydrolytic stability, infrared stretching frequencies, and Hammett's sigma values (56). In the structure shown below in which Y is varied, the lability of the P—O bond is directly related to the substituent Y.



The effects of substituents (Y) on the reactivity of the P—O bond can be correlated by a general quantitative relationship known as the Hammett equation:

$$\log k/k_0 = \sigma\rho \qquad (15)$$

Here $k$ and $k_0$ are rate or equilibrium constants for reactions of the substituted and unsubstituted compounds, respectively, $\sigma$ is the substituent constant which depends solely on the nature and position of Y, and $\rho$ is the reaction constant, which depends on the reaction and the conditions under which it takes place. The validity of this equation is restricted to *meta*- and *para*-positions of the benzene ring. The practical significance of the sigma values has been described in a preceding section; briefly, electron-releasing groups have negative values and electron-withdrawing groups have positive values. The sigma

TABLE III

Biological Activity and Chemical Properties of Diethyl Substituted Phenyl Phosphates

| Diethyl substituted phenyl phosphates | $I_{50}$ Fly-brain AChE (mol. conc.) | $LD_{50}$ Housefly ($\gamma$/g.) | $LD_{50}$ Thrips (% conc.) | $LD_{50}$ Mites (% conc.) | P—O—Ar stretching frequency, wave no. (cm.$^{-1}$) | First order $K_{hyd}$ (min.$^{-1}$) pH 9.5, 37°C. |
|---|---|---|---|---|---|---|
| 2,4-di-$NO_2$ | $3.0 \times 10^{-9}$ | 155 | 0.01 | 0.01 | 1265 | $5.7 \times 10^{-3}$ |
| *p*-$NO_2$ | $2.6 \times 10^{-8}$ | 0.5 | 0.0001 | 0.001 | 1240 | $2.7 \times 10^{-4}$ |
| *o*-$NO_2$ | $5.0 \times 10^{-8}$ | 7.0 | 0.001 | 0.003 | — | — |
| *m*-$NO_2$ | $5.0 \times 10^{-8}$ | 9.8 | 0.005 | 0.03 | 1230 | $9.8 \times 10^{-5}$ |
| 2,4,5-tri-Cl | $6.0 \times 10^{-9}$ | 8.0 | 0.03 | 0.0003 | 1253 | $7.9 \times 10^{-5}$ |
| 2,4,6-tri-Cl | $3.3 \times 10^{-6}$ | 175 | 0.03 | 0.03 | — | — |
| 2,4-di-Cl | $5.0 \times 10^{-7}$ | 15.0 | 0.003 | 0.1 | — | $4.8 \times 10^{-5}$ |
| *o*-Cl | $2.0 \times 10^{-5}$ | 250 | 0.01 | >0.1 | — | $5.1 \times 10^{-5}$ |
| *p*-Cl | $3.0 \times 10^{-5}$ | 150 | 0.01 | >0.1 | 1217 | $3.2 \times 10^{-5}$ |
| *p*-$^+S(CH_3)_2 \cdot CH_3SO_4^-$ | $3.4 \times 10^{-9}$ | 17.5 | 0.0002 | 0.001 | — | — |
| *p*-$SO_2CH_3$ | $2.5 \times 10^{-7}$ | 2.5 | 0.0001 | 0.001 | 1226 | — |
| *p*-$SOCH_3$ | $3.1 \times 10^{-6}$ | 1.5 | 0.0001 | 0.0008 | 1223 | — |
| *p*-$SCH_3$ | $3.3 \times 10^{-5}$ | 2.0 | 0.0001 | 0.0002 | 1218 | — |
| *m*-$^+N(CH_3)_3I^-$ | $3.0 \times 10^{-8}$ | >500 | >0.1 | >0.1 | — | — |
| *m*-$N(CH_3)_2$ | $4.0 \times 10^{-7}$ | 25 | 0.1 | 0.1 | — | $1.9 \times 10^{-6}$ |
| *p*-CN | $1.3 \times 10^{-7}$ | 3.5 | 0.00002 | 0.002 | 1241 | — |
| *m*-$OCH_3$ | $1.3 \times 10^{-4}$ | >500 | 0.02 | 0.3 | 1200 | $8.9 \times 10^{-6}$ |
| *p*-$OCH_3$ | $>1.0 \times 10^{-3}$ | >500 | 1.0 | 1.0 | 1205 | — |
| *m*-*t*-butyl | $9.0 \times 10^{-7}$ | 500 | 0.003 | >0.1 | 1207 | $8.6 \times 10^{-6}$ |
| *p*-*t*-butyl | $1.0 \times 10^{-4}$ | >500 | 0.1 | >0.1 | — | — |
| *p*-$CH_3$ | $>1.0 \times 10^{-3}$ | >500 | >0.1 | >0.1 | 1219 | — |
| H | $>1.0 \times 10^{-3}$ | >500 | >0.1 | >0.1 | 1215 | $9.2 \times 10^{-6}$ |
| *p*-CHO | $1.5 \times 10^{-7}$ | >500 | 0.05 | 0.05 | 1229 | — |
| *p*-COOH | $8.5 \times 10^{-7}$ | 150 | 0.005 | 0.01 | 1227 | — |

Data from (56).

values of a wide variety of substituents have been calculated and are listed in a review article (76).

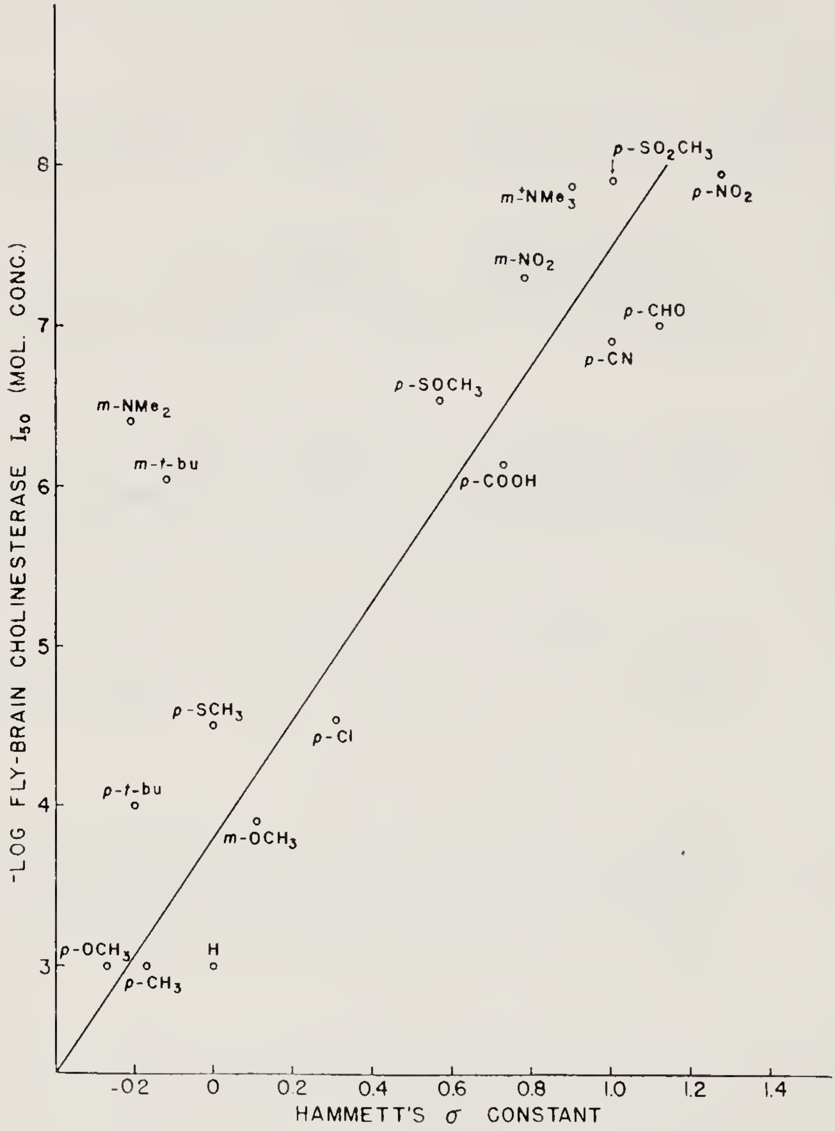


Fig. 3. Relationship between $-\log$ fly-brain $I_{50}$ versus Hammett's sigma constants (56).

The hydrolysis of diethyl substituted phenyl phosphates has been shown to conform to the Hammett equation (76,56), and a linear relationship is obtained when the log of the hydrolysis constants are plotted against the sigma values of the substituents. Since erythrocyte AChE inhibition by the diethyl substituted phenyl phosphates has also been shown to be a function of the hydrolysis constants (see

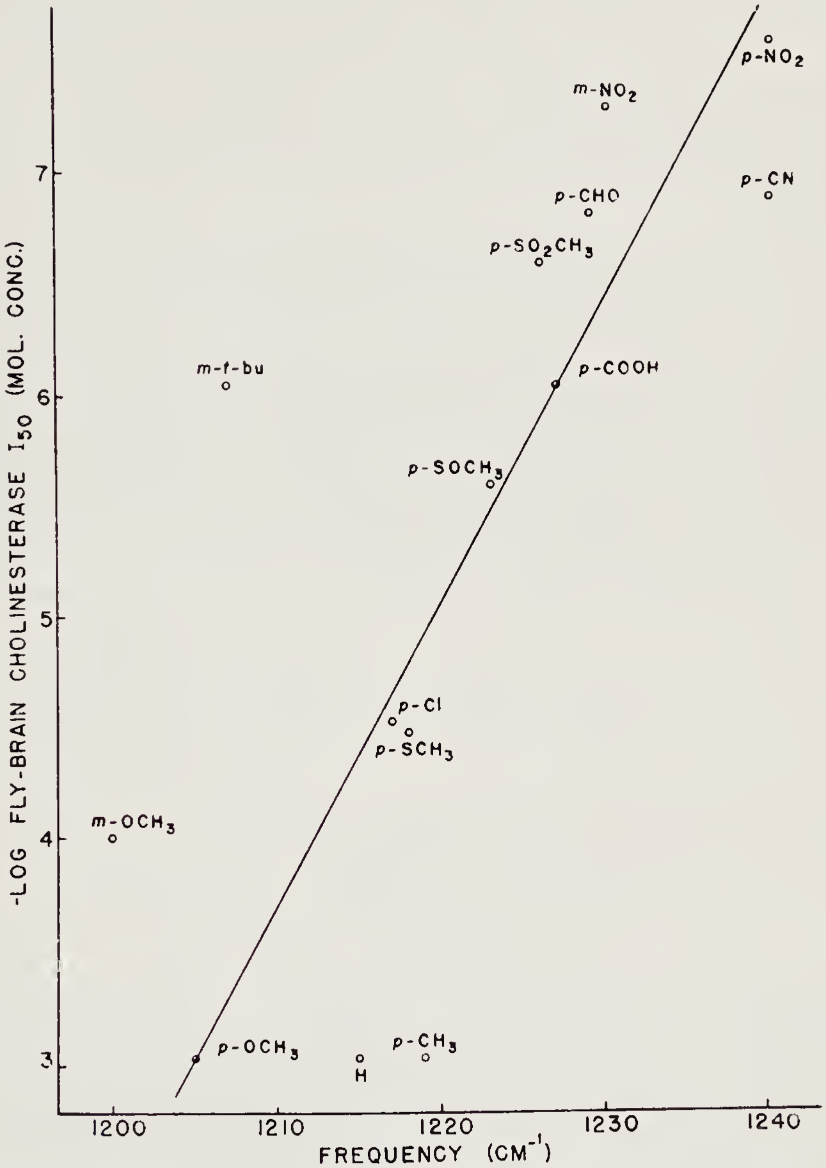


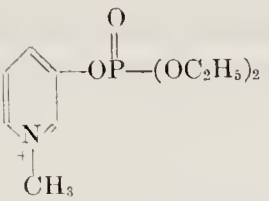
Fig. 4. Relationship between $-\log$ fly-brain AChE $I_{50}$ versus P—O—C (aromatic) stretching frequency (56).

Fig. 2), it necessarily follows that the degree of enzyme inhibition should also be a function of the sigma values. When the negative log of the molar concentration for 50% inhibition of fly-brain AChE ($-\log I_{50}$) by a series of diethyl substituted phenyl phosphates is plotted against the corresponding sigma values, a straight-line relationship is obtained as demonstrated in Fig. 3. It is seen that the degree of enzyme inhibition increases as the substituents in the benzene ring become more electron-attracting as measured by the sigma constants.

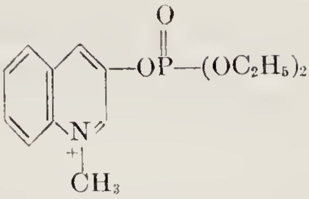
As a corollary to Hammett's treatment the infrared spectra of a number of diethyl substituted phenyl phosphates have been determined and the shifts in the P—O—C (aromatic) stretching frequency examined. The frequencies of this bond and the corresponding fly-brain AChE $I_{50}$ values given in Table III show that the wave number is also a function of the electron-releasing or withdrawing property of the substituent and parallels the degree of fly-brain AChE inhibition. A plot of $-\log I_{50}$ against frequency is similar to that obtained using sigma values as shown in Fig. 4.

It is significant from Figs. 3 and 4 that for any given series of dialkyl substituted phenyl phosphates, the inhibitory activities may be predicted from sigma values which are available in the literature, and by the P—O—Ar stretching frequencies which are readily obtained.

Examination of Figs. 3 and 4, and the first order hydrolysis constants, $K_{\text{hyd}}$, listed in Table III, show that *m-t*-butylphenyl and *m*-dimethylaminophenyl diethyl phosphates are far more inhibitory than predicated on the effect of the *m-t*-butyl and *m*-dimethylamino substituents on the lability of the P—O bond. This apparent anomaly is strong evidence for the hypothesis already discussed in a preceding section that the inhibition of cholinesterase by many of the organic phosphates is influenced by steric attraction in which the inhibitor is brought into close proximity to the active sites before reacting with the enzyme. In addition to those already mentioned in the previous section the methosulfates of diethyl 3-quinolyl phosphate (IX) and diethyl 3-pyridyl phosphate (X) fall into this category and are among the most powerful inhibitors of cholinesterase known, the former showing one of the highest *in vitro* anticholinesterase activities for an organic phosphate recorded in the literature so far. Their molar concentrations for 50% inhibition of erythrocyte AChE are $1.5 \times 10^{-10}\,M$ and $2.0 \times 10^{-8}\,M$, respectively (9,72).



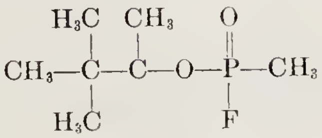
(X)



(IX)

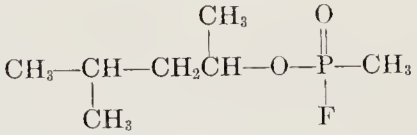
These two compounds and their analogues are of particular interest in that not only do their structures suggest good fit on the enzyme surface but the positively charged nitrogen will attract electrons from the phosphate moiety, thereby inducing electrophilic character in the phosphorus atom.

Enhancement of cholinesterase inhibition by steric attraction may also be obtained by fixing the configuration of one of the stable groups in the molecule. For example, the series of compounds exemplified by 3,3-dimethyl-2-butyl methylphosphonofluoridate (XI, soman), 1-methylisoamyl methylphosphonofluoridate (XII), and isopropyl methylphosphonofluoridate (XIII, sarin) are considerably more potent as cholinesterase inhibitors than either tabun or DFP (126).



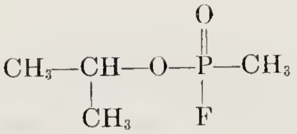
$$CH_3-\overset{H_3C}{\underset{H_3C}{C}}-\overset{CH_3}{C}-O-\overset{\overset{O}{\|}}{\underset{F}{P}}-CH_3$$

(XI)



$$CH_3-\underset{CH_3}{CH}-CH_2\overset{CH_3}{CH}-O-\overset{\overset{O}{\|}}{\underset{F}{P}}-CH_3$$

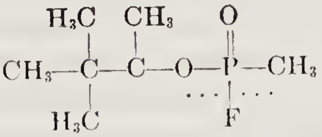
(XII)



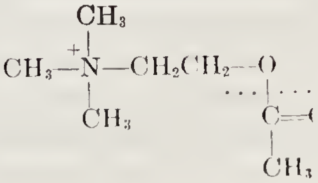
$$CH_3-\underset{CH_3}{CH}-O-\overset{\overset{O}{\|}}{\underset{F}{P}}-CH_3$$

(XIII)

Of the series, soman was found to be far superior as an inhibitor over the others. The structural similarity between soman and acetylcholine is apparent from the illustrations below and strongly suggests that steric attraction is an important factor in the inhibition process.

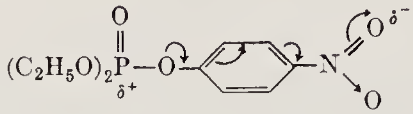


$$CH_3-\overset{H_3C}{\underset{H_3C}{C}}-\overset{CH_3}{C}-O-\overset{\overset{O}{\|}}{\underset{F}{P}}-CH_3$$

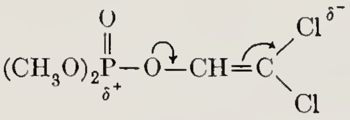


$$CH_3-\overset{CH_3}{\underset{CH_3}{\overset{+}{N}}}-CH_2CH_2-\underset{\underset{CH_3}{C=O}}{O}$$

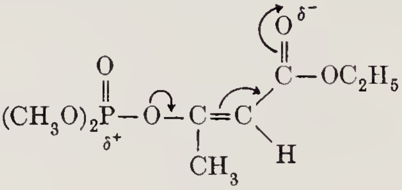
Substituents with highly positive sigma values probably induce electrophilic character to the phosphorus atom by a shift of electrons exemplified below with para-oxon.



It is reasonable to suspect, therefore, that compounds containing an unsaturated group with substituents of positive sigma values in conjugation with the P—O—C bond should show inhibitory activity. This has been shown to be the case with a large number of dialkyl substituted vinyl phosphates, including *O,O*-dimethyl *O*-2,2-dichlorovinyl phosphate (DDVP) (28,90) and *O,O*-dimethyl *O*-1-carbomethoxy-1-propen-2-yl phosphate (OS-2046) (37,8) which are exceedingly potent inhibitors of cholinesterase.



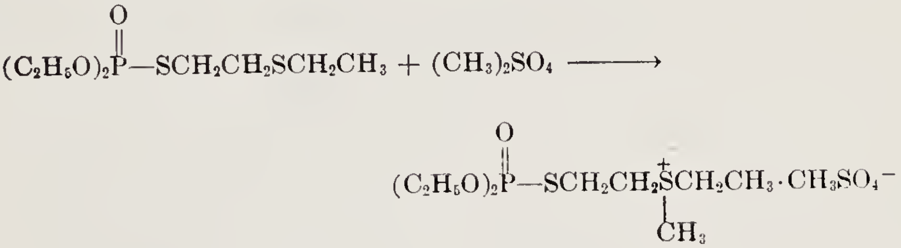
DDVP



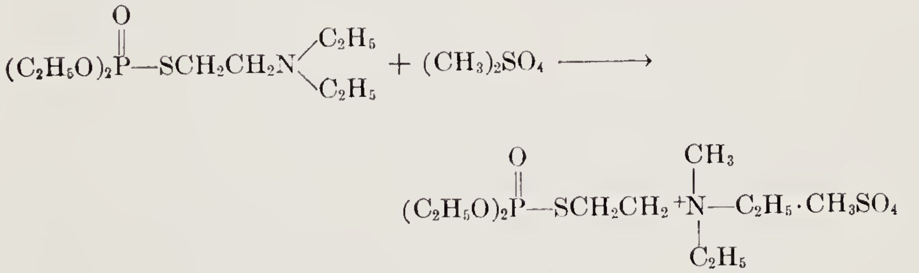
OS–2046

Examination of the biological data in Table III shows that, in general, there is good correlation between enzyme inhibition and insect toxicity. There are, however, a number of exceptions where insect toxicities are not in line with *in vitro* AChE inhibition and which is not surprising in view of the complex nature of living tissues. The relatively low toxicity of diethylaminophenyl phosphate methiodide and diethyl *p*-methylmercaptophenyl phosphate methosulfate, although potent inhibitors, would suggest that penetration through the insect cuticle or lipoid nerve sheaths is an important factor and that polar compounds (ions) are poor in this respect. It has been shown that the exposed nerve of the cockroach *Periplaneta americana*, in which the nerve sheath remains intact is not capable of hydrolyzing ionized substrates such as acetylcholine and $\beta$-methylacetylcholine which carry permanent positive charges (119) but does hydrolyze unionized substrates such as ethyl acetate, triacetin, and phenyl acetate, suggesting that ionized substrates cannot penetrate the nerve sheath.

The problem of penetration through the insect cuticle or lipoid nerve sheath by ions is further illustrated by comparing the relative toxicities of *O,O*-diethyl *S*-ethyl-2-mercaptoethyl phosphorothiolate (thiol isomer of Systox) with its methosulfate salt, and *O,O*-diethyl *S*-2-diethylaminoethyl phosphorothiolate (R-6199) with its methosulfate salt. The methosulfates are conveniently prepared by treating the original compounds with dimethyl sulfate, producing the permanently charged sulfonium and quaternary ammonium salts.



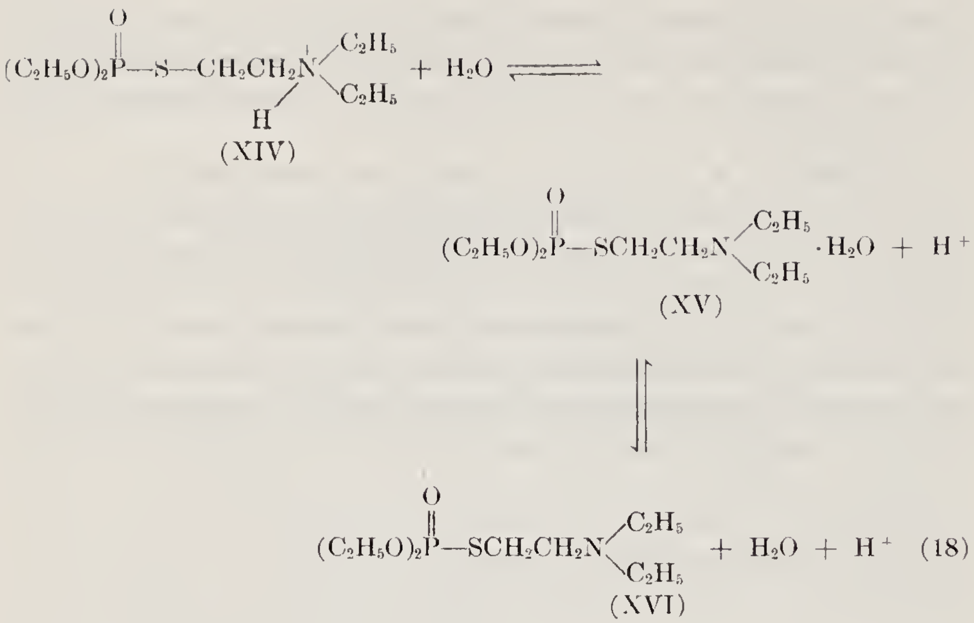
$$(C_2H_5O)_2\overset{O}{\overset{\|}{P}}\text{—}SCH_2CH_2SCH_2CH_3 + (CH_3)_2SO_4 \longrightarrow (C_2H_5O)_2\overset{O}{\overset{\|}{P}}\text{—}SCH_2CH_2\underset{CH_3}{\overset{+}{S}}CH_2CH_3\cdot CH_3SO_4^- \tag{16}$$



$$(C_2H_5O)_2\overset{O}{\overset{\|}{P}}\text{—}SCH_2CH_2N\begin{matrix} C_2H_5 \\ C_2H_5 \end{matrix} + (CH_3)_2SO_4 \longrightarrow (C_2H_5O)_2\overset{O}{\overset{\|}{P}}\text{—}SCH_2CH_2\,{}^{+}\underset{C_2H_5}{\overset{CH_3}{N}}\text{—}C_2H_5\cdot CH_3SO_4 \tag{17}$$

With the thiol isomer, conversion to the methosulfate increases the AChE inhibitory activity 100-fold, from an $I_{50}$ of $3.0 \times 10^{-6}\ M$ to $3.3 \times 10^{-8} M$; the activities of R-6199 and its methosulfate are about equal and in the order of $3 \times 10^{-9} M$ (59,64,61). The comparative contact toxicities of the methosulfates, to various insects, however, is much lower than the parent compounds and can only be attributed to slow penetration of the positively charged ions.

In this regard, R-6199, which is now undergoing rigorous evaluation as an insecticide in the form of its oxalate salt, is capable of equilibrating with water to a number of forms as shown (118):



$$(C_2H_5O)_2\overset{O}{\overset{\|}{P}}-S-CH_2CH_2\overset{+}{N}H(C_2H_5)_2 + H_2O \rightleftharpoons$$

(XIV)

$$(C_2H_5O)_2\overset{O}{\overset{\|}{P}}-SCH_2CH_2N(C_2H_5)_2\cdot H_2O + H^+$$

(XV)

$$\rightleftharpoons (C_2H_5O)_2\overset{O}{\overset{\|}{P}}-SCH_2CH_2N(C_2H_5)_2 + H_2O + H^+ \quad (18)$$

(XVI)

The equilibria shown are typical of tertiary ammonium salts in which (XIV) is hydrolyzed by water to give an unionized hydrated amine (XV), which in turn is in equilibrium with the free amine (XVI). The penetration of (XIV) through insect tissue may, therefore, be attributed to the formation of (XV) and (XVI). After penetration, equilibrium is immediately reestablished and the toxic action is probably due to (XIV), which as discussed earlier is expected to be the most potent inhibitor among the three forms.

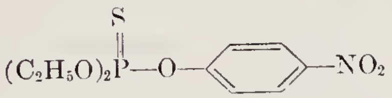
Moderate stability to aqueous hydrolysis is another factor which must be considered and the low toxicity of diethyl 2,4-dinitrophenyl phosphate (Table III) is probably due to hydrolytic degradation before it reaches the site of action. The second order rate constants, $K_{\mathrm{hyd}}$, show that it is about 21 times less stable at pH 9.5 than paraoxon. This factor is especially important in systemic insecticides which are constantly subjected to hydrolytic action by plant juices. TEPP, although water miscible, shows no systemic action probably because it is too rapidly hydrolyzed to the nontoxic diethyl phosphoric acid.

The unusually high or low insecticidal toxicity of some of the compounds relative to their *in vitro* enzyme inhibition shown in Table III suggest metabolic conversion to more or less toxic substances. The general subject of the metabolism of organophosphorus compounds in plants, insects, and mammals is discussed in detail in the next section.

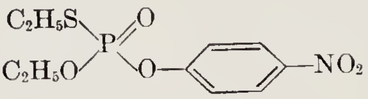
## V. Metabolism in Biological Systems

Studies on the metabolism and the fate of the organophosphorus insecticides in insects, animals, and plants have been essential in the elucidation of the mode of action of these compounds. Often the correlation between *in vitro* cholinesterase inhibition and insect toxicity is poor. For example, the phosphorothionates and phosphorodithioates are generally poor inhibitors of cholinesterase but are often quite toxic to insects and some of them, e.g., parathion and malathion, rank among the most important commercial insecticides today. This apparent anomaly between poor enzyme inhibition and high insect toxicity has been explained on findings that these compounds are metabolized in many biological systems to highly potent inhibitors.

Pure parathion has been demonstrated to be almost inactive as a cholinesterase inhibitor (45,104) and the anticholinesterase activity of the technical material has been attributed to the presence of the



Parathion



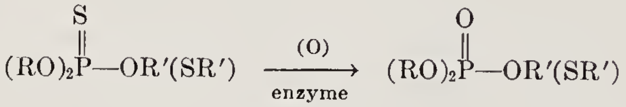
*S*-ethyl isomer

*S*-ethyl isomer. Pure samples of the dimethyl and di*iso*propyl analogues of parathion, ethyl *p*-nitrophenyl benzenephosphonothionate (EPN), malathion, and numerous other phosphorothionates and dithioates, although potent insecticides, have been shown to be rather weak cholinesterase inhibitors. The theoretical basis for the weak inhibitory activity of the thionates and dithioates has been discussed in the previous section.

In view of these facts, it appears that metabolic transformation of the P=S compound to an active anticholinesterase agent occurs in the animal. By incubating parathion with the intact nerve cord of *Periplaneta americana* the formation of an active substance was demonstrated (41). A more detailed study using tissues of *Periplaneta americana* showed that the conversion of pure parathion and methyl parathion to highly active cholinesterase inhibitors occurs only with intact tissues and when oxygen was present (103,109). It was also shown by means of paper chromatographic techniques that para-oxon and methyl para-oxon are produced by incubating parathion and methyl parathion, respectively, with the roach gut.

The metabolic conversion of parathion to para-oxon has also been demonstrated in various tissues of the rat (63,84). A comparison of the relative inhibitory potencies toward the AChE and ali-esterase activity of rat brain *in vitro* and *in vivo* showed that the inhibitory behavior of parathion and para-oxon were similar and different from that of the *S*-ethyl isomer, indicating that the inhibitory activity of parathion *in vivo* must be due largely to para-oxon formed by enzymic oxidation of P═S to P═O and not to the *S*-ethyl isomer (113).

The metabolic conversion of phosphorothionates and dithioates may be depicted as:



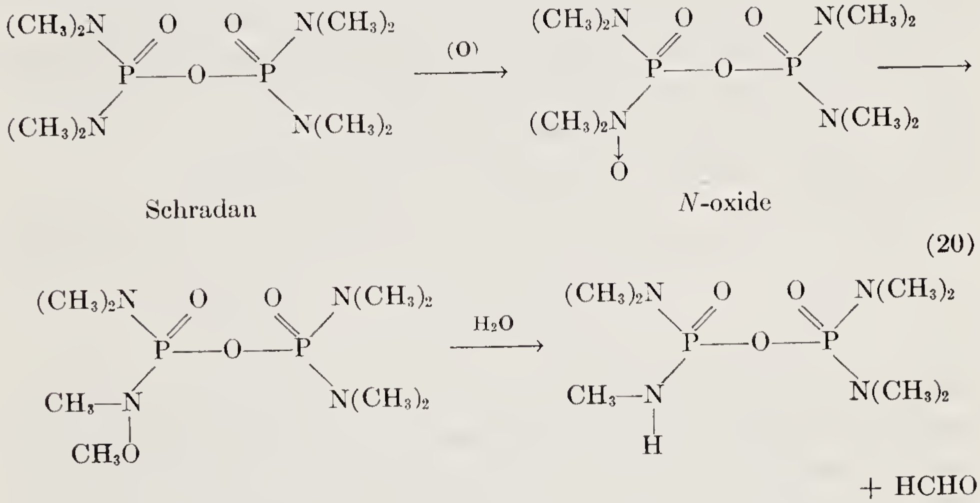
$$(RO)_2\overset{S}{\overset{\|}{P}}\text{—}OR'(SR') \xrightarrow[\text{enzyme}]{(O)} (RO)_2\overset{O}{\overset{\|}{P}}\text{—}OR'(SR') \qquad (19)$$

This enzymatic conversion of P═S to P═O has also been shown to occur with methyl parathion, malathion, EPN, *O,O*-diethyl *S*-ethylmercaptomethyl phosphorodithioate, and other P═S containing insecticides (103,31). In view of the wide variety of compounds which have been shown to undergo this conversion it is probably safe to assume that all P═S containing compounds behave similarly in certain biological systems. This does not necessarily imply that the toxic action of phosphorothionates and dithioates is due entirely to this metabolic activation. However, in cases where the thionate or dithioate itself is unable to act as an enzyme inhibitor, then enzymatic activation is probably essential in the poisoning process.

Schradan (or OMPA) as described earlier, is extremely stable to basic hydrolysis and also shows little or no anticholinesterase activity, e.g., an $I_{50}$ concentration value of greater than $1 \times 10^{-3}M$ (120, 83,39). Schradan is, however, highly toxic to certain insects but shows rather marked species specificity in that it is almost nontoxic to others (120,121,125).

The poisoning process in insects and mammals has been shown to involve the oxidative metabolism of Schradan to a highly active anticholinesterase. This metabolism has been demonstrated in a wide variety of biological tissues in several susceptible insects, including *Anasa tristis* and *Oncopeltus Fasciatus*, and nonsusceptible insects, *Periplaneta americana* and *Tenebrio molitor* L. (120,36). In mammals, metabolism appears to occur principally in the liver (43). Metabolism of Schradan to a more active inhibitor has also been shown to occur in plants (39,50) and the initial product is probably similar to that obtained in insect and mammalian metabolism. However, plant metabolism appears to take place very slowly and the metabolite is probably hydrolytically destroyed as soon as it is formed.

The metabolic oxidation of Schradan appears to involve several products. The initial metabolite which inhibits ChE at $3.5 \times 10^{-7}M$, has been shown rather conclusively, by infrared spectra and comparison of chemical and biological properties, to be the *N*-oxide structure as shown in equation (20) (39,136,137,35) which subsequently rearranges to the *N*-methoxide structure.



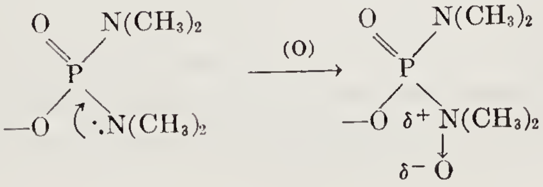
Schradan

*N*-oxide

(20)

*N*-methoxide

The *N*-oxide structure was originally proposed by early investigators (50,67) before its actual confirmation in order to account for the increase in reactivity of the anhydride bond. Since the lability of the P—O bond is a function of the electrophilic character of the phosphorus atom, the conversion of the electron-releasing dimethylamino moiety to the electron-attracting *N*-oxide structure will greatly enhance reactivity. As shown in equation (21) the electrophilic

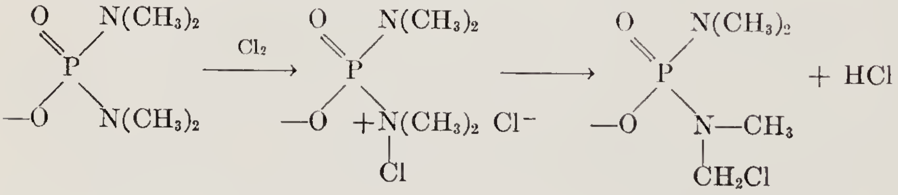


(21)

character of the phosphorus atom is weakened by the pair of electrons on the amino nitrogen; the formation of the *N*-oxide, however, removes this source of electrons and in addition creates a nitrogen atom with a positive formal charge which will attract electrons from the phosphorus atom by an induction mechanism.

The oxidation of Schradan to the *N*-oxide has also been effected by treatment with permanganate, dichromate, hypochlorite, and bromine water (136). The *N*-oxide obtained from both chemical and biological oxidation has been shown to rearrange readily to the *N*-methoxide structure (136,131) under slightly alkaline conditions or by heating. The *N*-methoxide is more stable to hydrolysis and a weaker anticholinesterase than the *N*-oxide, and can be decomposed under acidic conditions to formaldehyde and the mono-demethylated Schradan.

A similar activation of Schradan has been demonstrated by treatment with anhydrous chlorine in which a highly electronegative chlorine atom is introduced into the molecule.



(22)

The $I_{50}$ for human serum ChE was $2 \times 10^{-7}M$ for the mono-chloro derivative, an increase of $10^5$-fold in potency over the original Schradan. It was found that as many as four chlorine atoms could be introduced but these polychlorinated derivatives were, in general, much too unstable under aqueous conditions to effect inhibition of ChE (131).

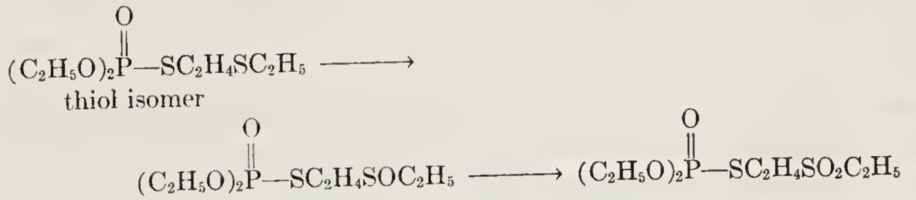
The marked species specificity of Schradan has been attributed by some investigators to inability of the metabolite to penetrate the lipoid nerve sheath in certain insects and that in these insects Schradan is rapidly converted in the body fat before reaching the nerve tissue (120). Other investigators feel that the specificity difference is due to the sensitivity of the particular insect ChE to inhibition by the metabolite (36).

The chemical and metabolic conversion of tetramethylphosphorodiamidofluoridate and *p*-nitrophenyl tetramethylphosphorodiamidate, which are both weak anticholinesterases, to highly active inhibitors has also been demonstrated (34,67).

Particular attention has been devoted to the study of the metabolism of systemic insecticides which are often metabolized in plants to substances more toxic than the original insecticide, making it necessary to determine the nature of the metabolic products, particularly in regard to residual hazards.

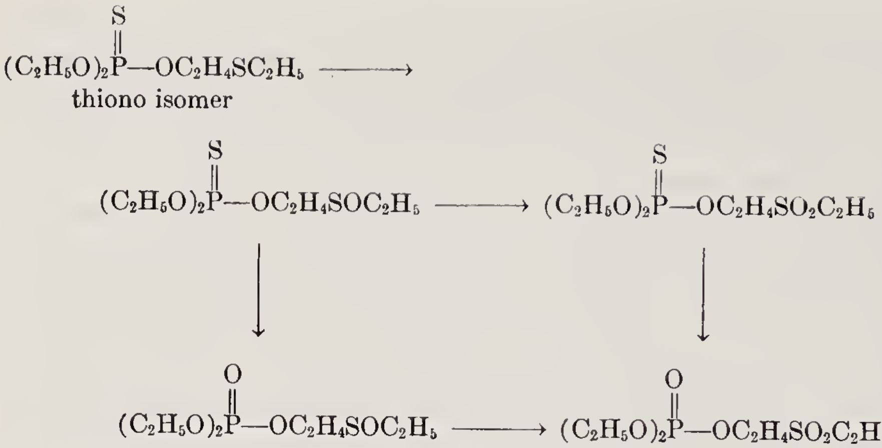
The systemic insecticide, Systox, is a mixture of two isomers, *O,O*-diethyl *O*-ethyl-2-mercaptoethyl phosphorothionate (thiono isomer) and *O,O*-diethyl *S*-ethyl-2-mercaptoethyl phosphorothiolate (thiol isomer). The thiono isomer is easily converted with heat to the thiol isomer and the kinetics of this rearrangement have been shown to be first order (58,71). The thiono isomer is considerably more stable than the thiol isomer to alkaline hydrolysis and the $I_{50}$ for serum ChE is $10^{-5}M$ for the former and $3 \times 10^{-6}M$ for the latter. This difference in cholinesterase inhibition is evident in the greater mammalian and insect toxicity of the thiol isomer (69).

The two isomers are rapidly absorbed by a wide variety of plants and after a few days essentially none of the original isomers can be found in the plant (69,108). The presence of at least three different metabolites for each of the isomers was indicated by counter-current partition techniques. A more detailed study of the metabolism in cotton plants indicated that these isomers were converted to certain oxidation products that were even more potent inhibitors of cholinesterase than the parent isomers. Comparison of biological and chemical properties, and relative behavior on paper chromatograms indicated that each isomer is first converted to the corresponding sulfoxide by oxidation of the ethylmercaptoethyl moiety, and suggested that these sulfoxides were subsequently converted to the sulfones (60). The sequence of reactions probably involved in the metabolism is illustrated in equations (23) and (24).



$$\underset{\text{thiol isomer}}{(C_2H_5O)_2\overset{O}{\overset{\|}{P}}-SC_2H_4SC_2H_5} \longrightarrow (C_2H_5O)_2\overset{O}{\overset{\|}{P}}-SC_2H_4SOC_2H_5 \longrightarrow (C_2H_5O)_2\overset{O}{\overset{\|}{P}}-SC_2H_4SO_2C_2H_5 \qquad (23)$$

A similar study of the metabolism of the two isomers in the white mouse and *Periplaneta americana* showed that the routes of metabolism and the toxic metabolites formed are the same in plants and animals (93).



$$\begin{tikzcd}[column sep=small]
\underset{\text{thiono isomer}}{(C_2H_5O)_2\overset{S}{\overset{\|}{P}}\text{—}OC_2H_4SC_2H_5} \arrow[r] & {} \\
(C_2H_5O)_2\overset{S}{\overset{\|}{P}}\text{—}OC_2H_4SOC_2H_5 \arrow[r] \arrow[d] & (C_2H_5O)_2\overset{S}{\overset{\|}{P}}\text{—}OC_2H_4SO_2C_2H_5 \arrow[d] \\
(C_2H_5O)_2\overset{O}{\overset{\|}{P}}\text{—}OC_2H_4SOC_2H_5 \arrow[r] & (C_2H_5O)_2\overset{O}{\overset{\|}{P}}\text{—}OC_2H_4SO_2C_2H_5
\end{tikzcd} \qquad (24)$$

The infrared spectra of the metabolites of thiol isomer isolated from the cotton plant proved conclusively that the major metabolite is the thiol-isomer sulfoxide; the thiol-isomer sulfoxide is then metabolized further to the sulfone but at a much slower rate than the conversion of the thiol isomer to the sulfoxide (62).

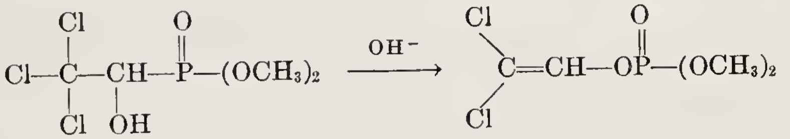
Other organophosphorus compounds containing a sulfide linkage have been shown to undergo this type of metabolism. *O,O*-Diethyl *S*-ethylmercaptomethyl phosphorodithioate (31) and *O,O*-diethyl *S*-ethyl-2-mercaptoethyl phosphorodithioate (100) are also converted to the sulfoxides and sulfones in plants.

The order in which the various oxidation products of each of the two isomers of Systox inhibit fly-brain AChE was found to be sulfone > sulfoxide > sulfide. This is in line with the predicted order of the reactivity of the P—O and P—S bonds on the basis that sulfones are more electron-attracting than sulfoxides, which in turn are more electron-attracting than sulfides.

The relatively high toxicity of *p*-methylmercaptophenyl and *p*-methylsulfinylphenyl diethyl phosphate as compared to *p*-methylsulfonylphenyl diethyl phenyl phosphate is readily explained by oxidation of the methylmercapto moiety to the sulfoxide and then to the sulfone. The data in Table III show that all three compounds are equally toxic to insects and mites although there is a 10-fold difference in their inhibition of fly-brain AChE.

The enzymatic hydrolysis of various organophosphorus compounds in which a bond directly connected to the phosphorus atom is split has already been discussed in an earlier section. Enzymatic hydrolysis of other functional groups in the organophosphorus molecule also occurs. The detoxification mechanism of malathion, *O,O*-dimethyl *S*-1,2-dicarboethoxyethyl phosphorodithioate, appears to involve the enzymatic hydrolysis of one or both of the carboethoxy groups, although the complete metabolic picture in both insects and mammals is probably more complex as indicated by the large number of different metabolites detected (91,92). The degradation of the *cis* and *trans* isomers of *O,O*-dimethyl *O*-2-carbomethoxy-1-propen-2-yl phosphate in plants appears to be initiated by the enzymatic hydrolysis of the carbomethoxy moiety followed by cleavage of the vinyl phosphate bond (38).

Of particular interest because of its unusual properties, is the insecticide *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (Dipterex). Dipterex shows low-toxicity to mammals and although relatively nontoxic as a contact insecticide to certain insects it is highly toxic as a stomach poison and is now being sold commercially as a fly bait. It is readily dehydrochlorinated by alkaline treatment and rearranges to *O,O*-dimethyl *O*-2,2-dichlorovinyl phosphate, a highly potent inhibitor of fly-brain AChE (28,90) and a highly toxic contact poison.



$$\mathrm{Cl{-}CCl_2{-}CH(OH){-}P(=O){-}(OCH_3)_2} \xrightarrow{\mathrm{OH^-}} \mathrm{Cl_2C{=}CH{-}OP(=O){-}(OCH_3)_2} \qquad (25)$$

In view of the ability of many insects to dehydrochlorinate compounds containing the trichloroethyl moiety such as DDT and its analogues, it has been speculated that metabolism to the vinyl phosphate occurs *in vivo* in these insects where Dipterex is toxic (24). Studies with insects, plants, and mammals have shown, however, that this does not appear to be the case and metabolic degradation involving the direct cleavage of the P—C bond takes place (10).

## VI. Summary

The preceding discussions have made it clear that high anticholinesterase activity is not an essential prerequisite for high insecticidal activity. In synthesizing a new organophosphorus insecticide, however, it is necessary to keep in mind that the compound must inactivate cholinesterases either directly, or indirectly as occurs through a metabolic intermediate. To summarize briefly, organophosphorus compounds of high anticholinesterase activity may be divided into two distinct groups. Compounds in the first group are those containing one, and only one, reactive bond connected to the phosphorus atom, or which can be metabolized by an animal or plant to give such a compound. Compounds in the second group are those which resemble the natural substrate, acetylcholine, either by its overall configuration or by the presence of a cationic site situated at a precise distance from the ester moiety. In certain cases the requisites of both groups may be combined to produce an extremely potent inhibitor.

Insecticides of high mammalian toxicity, such as parathion, are being widely used and although we are aware of the hazards of these poisons, we are certain that they may be handled safely in insect control. However, the ideal insecticide is, of course, still one which is specific only to insects, exemplified by malathion, Dipterex, and others. These compounds are our link to better and more specific insecticides and it is essential that we investigate their metabolism and chemical behavior in insects and in mammals in complete detail.

## References

(1) Adams, D., and Whittaker, V., *Biochim. et Biophys. Acta*, *3*, 358 (1949).
(2) Aldridge, W., *Biochem. J.*, *53*, 110 (1953).
(3) Aldridge, W., *Biochem. J.*, *46*, 451 (1950).
(4) Aldridge, W., *Biochem. J.*, *53*, 62 (1953).
(5) Aldridge, W., *Biochem. J.*, *53*, 117 (1953).
(6) Aldridge, W., and Davison, A., *Biochem. J.*, *51*, 62 (1952).
(7) Aldridge, W., and Davison, A., *Biochem. J.*, *55*, 763 (1953).
(8) Allen, J., and Johnson, O., *J. Am. Chem. Soc.*, *77*, 2871 (1905).
(9) Andrews, K., Atherton, F., Bergel, F., and Morrison, A., *J. Chem. Soc.*, *1954*, 1638.
(10) Arthur, B., and Casida, J., Entomol. Soc. America, 3rd Annual Meeting, Cincinnati, November, 1955.
(11) Augustinsson, K., *Acta Chem. Scand.*, *4*, 1149 (1950).
(12) Augustinsson, K., *Acta Chem. Scand.*, *4*, 536 (1950).
(13) Augustinsson, K., *Acta Physiol. Scand.*, *32*, 173 (1954).
(14) Augustinsson, K., *Acta Chem. Scand.*, *5*, 712 (1951).
(15) Augustinsson, K., *Acta Physiol. Scand. Suppl.*, *52*, 15 (1948).
(16) Augustinsson, K., *Acta Chem. Scand.*, *5*, 699 (1951).
(17) Augustinsson, K., *Arch. Biochem.*, *23*, 111 (1949).
(18) Augustinsson, K., in Sumner, J. B., and Myrbäck, K., *The Enzymes*, Vol. I, Academic Press, New York, 1950.
(19) Augustinsson, K., and Heimbürger, G., *Acta Chem. Scand.*, *8*, 762 (1954).
(20) Augustinsson, K., and Graber, M., *Acta Physiol. Scand.*, *27*, 10 (1950).

(21) Augustinsson, K., and Heimbürger, G., *Acta Chem. Scand.*, *8*, 753 (1954).
(22) Augustinsson, K., and Heimbürger, G., *Acta Chem. Scand.*, *8*, 1533 (1954).
(23) Averell, P., Norris, M., and Vail, A., *J. Agr. Food Chem.*, *2*, 570 (1954).
(24) Babers, F., and Mitlin, N., *J. Econ. Entomol.*, *48*, 430 (1955).
(25) Babers, F., and Pratt, J., Jr., *Physiol. Zool.*, *24*, 127 (1951).
(26) Bailly, O., and Gaume, J., *Bull. soc. chim. France*, *3*, No. 5, 1396 (1936).
(27) Baldridge, H., Jr., McCarville, W., and Friess, L., *J. Am. Chem. Soc.*, *77*, 739 (1955).
(28) Barthel, W., Alexander, B., Giang, P., and Hall, A., *J. Am. Chem. Soc.*, *77*, 2424 (1955).
(29) Blimmer, R., and Burch, W., *J. Chem. Soc.*, *1929*, 279, 292.
(30) Blumenthal, E., and Herbert, J., *Trans. Faraday Soc.*, *41*, 611 (1945).
(30a) Boursnell, J., and Webb, E., *Nature*, *164*, 875 (1949).
(31) Bowman, J., and Casida, J., Entomol. Soc. America, 3rd Annual Meeting, Cincinnati, November, 1955.
(32) Brauer, R., *J. Pharmacol. Exptl. Therap.*, *92*, 162 (1948).
(33) Casida, J., *Biochem. J.*, *60*, 487 (1955).
(34) Casida, J., Allen, T., and Stahmann, M., *Nature*, *172*, 243 (1953).
(35) Casida, J., Allen, T., and Stahmann, M., *J. Am. Chem. Soc.*, *74*, 5548 (1952).
(36) Casida, J., Chapman, R., Stahmann, M., and Allen, T., *J. Econ. Entomol.*, *47*, 64 (1954).
(37) Casida, J., Gatterdam, P., Getzin, L., and Chapman, R., *J. Agr. Food Chem.*, *4*, 236 (1956).
(38) Casida, J., Gatterdam, P., Getzin, P., and Chapman, R., *J. Agr. Food Chem.*, *4*, 236 (1956).
(39) Casida, J., and Stahmann, M., *J. Agr. Food Chem.*, *1*, 883 (1953).
(40) Chadwick, L., and Hill, D., *J. Neurophysiol.*, *10*, 235 (1947).
(41) Chamberlain, W., and Hoskins, W., *J. Econ. Entomol.*, *44*, 177 (1951).
(42) Chefurka, W., and Smallman, B., *Nature*, *175*, 946 (1955).
(43) Cheng, K., *Brit. J. Exptl. Pathol.*, *32*, 444 (1951).
(44) Davison, W., *Biochem. J.*, *60*, 339 (1955).
(45) Diggle, W., and Gage, J., *Biochem. J.*, *49*, 491 (1951).
(46) Dostrovsky, I., and Halmann, M., *J. Chem. Soc.*, *1953*, 502.
(47) Dostrovsky, I., and Halmann, M., *J. Chem. Soc.*, *1953*, 508.
(48) Dostrovsky, I., and Halmann, M., *J. Chem. Soc.*, *1953*, 511.
(49) Dostrovsky, I., and Halmann, M., *J. Chem. Soc.*, *1953*, 516.
(50) Dubois, K., Doull, J., and Coon, J., *J. Pharmacol. Exptl. Therap.*, *99*, 367 (1950).
(51) Epstein, J., Rosenblatt, D., and Demek, M., *J. Am. Chem. Soc.*, *78*, 341 (1956).
(52) Feldberg, W., *Physiol. Revs.*, *25*, 596 (1945).
(53) Fleisher, J., Jandorf, B., Summersen, W., and Norton, D., *Fed. Proc.*, *9*, 171 (1950).
(54) Friess, S., and McCarville, W., *J. Am. Chem. Soc.*, *76*, 1363 (1955).
(55) Friess, S., and McCarville, W., *J. Am. Chem. Soc.*, *76*, 2260 (1955).
(56) Fukuto, T., and Metcalf, R., *J. Agr. Food Chem.*, *4*, 930 (1956).

(57) Fukuto, T., and Metcalf, R., unpublished work.

(58) Fukuto, T., and Metcalf, R., *J. Am. Chem. Soc.*, *76*, 5103 (1954).

(59) Fukuto, T., Metcalf, R., March, R., and Maxon, M., *J. Am. Chem. Soc.*, *77*, 3670 (1955).

(60) Fukuto, T., Metcalf, R., March, R., and Maxon, M., *J. Econ. Entomol.*, *48*, 347 (1955).

(61) Fukuto, T., and Stafford, E., unpublished work.

(62) Fukuto, T., Wolf, J., Metcalf, R., March, R., *J. Econ. Entomol.*, *49*, 147 (1956).

(63) Gage, J., *Biochem. J.*, *54*, 426 (1953).

(64) Ghosh, R., and Newman, J., *Chemistry & Industry*, *1955*, 118.

(65) Gowron, O., Grelechi, C., and Duggan, M., *Arch. Biochem. Biophys.*, *44*, 455 (1953).

(66) Hammett, L., *Physical Organic Chemistry*, McGraw-Hill Book Co., New York, 1950.

(67) Hartley, B., 15th Intern. Chem. Congr., New York, 1951.

(68) Hartley, B., and Kilby, B., *Biochem. J.*, *50*, 672 (1952).

(69) Heath, D., *Proc., 3rd Intern. Congr. Crop Protection, Paris, 1952.*

(70) Heles, G., and Hawes, I., *J. Biol. Chem.*, *133*, 375 (1940).

(71) Henglein, A., and Schrader, G., *Z. Naturforsch*, *10б*, 12 (1955).

(72) Hobbiger, F., *Brit. J. Pharmacol.*, *9*, 159 (1954).

(73) Hopf, H., *Ann. Appl. Biol.*, *39*, 193 (1952).

(74) Hopf, H., *Ann. Appl. Biol.*, *41*, 248 (1954).

(75) Ingold, C., *Structure and Mechanism in Organic Chemistry*, Cornell University Press, Ithaca, New York, 1953.

(76) Jaffé, H., *Chem. Revs.*, *53*, 191 (1953).

(77) Jandorf, B., Wagner-Jauregg, T., O'Neill, J., and Stolberg, M., *J. Am. Chem. Soc.*, *74*, 1251 (1952).

(78) Jansen, E., and Balls, A., *J. Biol. Chem.*, *194*, 721 (1952).

(79) Jansen, E., Curl, A., and Balls, A., *J. Biol. Chem.*, *190*, 557 (1951).

(80) Jansen, E., Nutting, M., Jang, R., and Balls, A., *J. Biol. Chem.*, *179*, 189 (1949).

(81) Jansen, E., Nutting, M., and Balls, A., *J. Biol. Chem.*, *179*, 201 (1949).

(82) Jansen, E., Nutting, M., Jang, R., and Balls, A., *J. Biol. Chem.*, *185*, 209 (1950).

(83) Kilby, B., *Chemistry & Industry*, *1953*, 856.

(84) Kok, G., and Walop, J., *Biochim et Biophys. Acta*, *13*, 510 (1954).

(85) Kolbezen, M., Metcalf, R., and Fukuto, T., *J. Agr. Food Chem.*, *2*, 864 (1954).

(86) Langemann, H., *Helv. Physiol. et Pharmacol. Acta*, *2*, C17 (1944).

(87) Larsson, L., *Acta Chem. Scand.*, *6*, 1470 (1952).

(88) Lewis, S., *Nature*, *172*, 1004 (1953).

(89) Lord, K., and Potter, C., *Ann. Appl. Biol.*, *38*, 495 (1951).

(90) Lorenz, W., Henglein, A., and Schrader, G., *J. Am. Chem. Soc.*, *77*, 2554 (1955).

(92) March, R., unpublished work.

(93) March, R., Fukuto, T., Metcalf, R., and Maxon, M., *J. Econ. Entomol.*, *49*, 185 (1956).

(94) March, R., Metcalf, R., Fukuto, T., and Maxon, M., *J. Econ. Entomol.*, *48*, 355 (1955).

(95) Massart, J., in Sumner, J. B. and Myrbäck, K., *The Enzymes*, Vol. I, Part 1, Academic Press, New York, 1950.

(96) Mazur, A., *J. Biol. Chem.*, *164*, 271 (1946).

(97) Means, O., Jr., *J. Cellular Comp. Physiol.*, *20*, 319 (1942).

(98) Mendel, B., Mundell, D., and Rudney, H., *Biochem. J.*, *37*, 473 (1943).

(99) Mendel, B., and Rudney, H., *Biochem. J.*, *37*, 59 (1943).

(100) Metcalf, R., unpublished work.

(101) Metcalf, R., *Organic Insecticides*, Interscience Publishers, Inc., New York-London, 1955.

(102) Metcalf, R., and March, R., *J. Econ. Entomol.*, *42*, 721 (1949).

(103) Metcalf, R., and March, R., *Ann. Entomol. Soc. Amer.*, *46*, 63 (1953).

(104) Metcalf, R., and March, R., *J. Econ. Entomol.*, *46*, 288 (1953).

(105) Metcalf, R., March, R., and Fukuto, T., unpublished work.

(106) Metcalf, R., March, R., and Maxon, M., *Ann. Entomol. Soc. Amer.*, *48*, 222 (1955).

(107) Metcalf, R., and March, R., *J. Econ. Entomol.*, *43*, 670 (1950).

(108) Metcalf, R., March, R., Fukuto, T., and Maxon, M., *J. Econ. Entomol.*, *47*, 1045 (1954).

(109) Metcalf, R., and March, R., *Science*, *117*, 527 (1953).

(110) Michel, H., and Krop, S., *J. Biol. Chem.*, *190*, 119 (1951).

(111) Mounter, L., and Whittaker, V., *Biochem. J.*, *50*, 551 (1953).

(112) Myers, D., and Mendel, B., *Biochem. J.*, *53*, 16 (1953).

(113) Myers, D., Mendel, B., Gersmann, H., and Ketalaar, J., *Nature*, *170*, 805 (1952).

(114) Nachmansohn, D., *Yale J. Biol. and Med.*, *12*, 565 (1940).

(115) Nachmansohn, D., in Fulton, J., *Physiology of the Nervous System*, 3rd ed., Oxford University Press, New York, 1950.

(116) Nachmansohn, D., in Pincus, G., and Thimann, K., *The Hormones*, Vol. II, Academic Press, New York, 1950.

(117) Nachmansohn, D., and Rothenberg, M., *J. Biol. Chem.*, *158*, 653 (1945).

(118) Noyes, A., and Sherrill, M., *A Course of Study in Chemical Principles*, The Macmillan Company, New York, 1953.

(119) O'Brien, R., *Ann. Entomol. Soc. Amer.*, in press.

(120) O'Brien, R., and Spencer, E., *J. Agr. Food Chem.*, *1*, 946 (1953).

(121) O'Brien, R., and Spencer, E., *J. Agr. Food Chem.*, *3*, 56 (1955).

(122) Ormerod, W., *Biochem. J.*, *54*, 701 (1953).

(123) Pauling, L., *Nature of the Chemical Bond*, Cornell University Press, Ithaca, N. Y., 1944.

(124) Richards, A., and Cutkamp, L., *J. Cellular Comp. Physiol.*, *26*, 57 (1945).

(125) Ripper, W., Greenslade, R., and Hartley, C., *Bull. Entomol. Research*, *40*, 481 (1950).

(126) Roeder, K., and Kennedy, N., *J. Pharmacol. Exptl. Therap.*, *114*, 211 (1955).

(127) Roeder, K., Kennedy, N., and Sampson, E., *J. Neurophysiol.*, *10*, 1 (1951).

(128) Sawyer, C., and Everett, J., *Am. J. Physiol., 148,* 675 (1947).
(129) Schrader, G., BIOS *Final Rept. 1095,* 30 (1947).
(130) Schrader, G., *Angew. Chem.,* Monograph *No. 62* (1952).
(131) Spencer, E., and O'Brien, R., *J. Agr. Food Chem., 1,* 716 (1953).
(132) Sturge, L., and Whittaker, V., *Biochem. J., 47,* 518 (1950).
(133) Tobias, J., Kollros, J., and Sanit, J., *J. Cellular Comp. Physiol., 28,* 159 (1948).
(134) Toy, A., *J. Am. Chem. Soc., 70,* 3882 (1948).
(135) Toy, A., *J. Am. Chem. Soc., 72,* 2065 (1950).
(136) Tsuyuki, H., Stahmann, M., and Casida, J., *J. Agr. Food Chem., 3,* 922 (1955).
(137) Tsuyuki, H., Stahmann, M., and Casida, J., *Biochem. J., 59,* iv (1955).
(138) Wagner-Jauregg, T., and Hockley, B., *J. Am. Chem. Soc., 75,* 2125 (1953).
(139) Wagner-Jauregg, T., O'Neill, J., and Summerson, W., *J. Am. Chem. Soc., 73,* 5202 (1951).
(140) Waters, W., and deWorms, C., *J. Chem. Soc., 1949,* 926.
(141) Webb, E., *Biochem. J., 42,* 96 (1948).
(142) Whittaker, V., *Biochem. J., 54,* 660 (1953).
(143) Wilson, I., *J. Biol. Chem., 197,* 215 (1952).
(144) Wilson, I., *Biochim. et Biophys. Acta, 7,* 466 (1951).
(145) Wilson, I., and Bergmann, F., *J. Biol. Chem., 186,* 683 (1950).
(146) Wilson, I., and Bergmann, F., *J. Biol. Chem., 186,* 693 (1950).
(147) Wilson, I., and Ginsburg, S., *Biochim. et Biophys. Acta, 18,* 168 (1955).
(148) Wilson, I., and Meislich, E., *J. Am. Chem. Soc., 75,* 4628 (1953).

# MECHANISMS OF FUNGITOXICITY

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## I. Introduction

Roland Thaxter, Harvard's famed eccentric, entered the Graduate School determined to be a mycologist, even if he had to earn his living first as a family physician. He dropped medical school after one year, however, in favor of his fungi, despite Farlow's dire warnings of economic troubles ahead.

Suddenly, in the middle of his graduate work, Bordeaux mixture showed up in the United States, imported by the fireball, F. Lamson-Scribner of the United States Department of Agriculture. Bordeaux mixture changed the whole course of plant pathology. It made possible the horde of plant doctors that roam the countryside these days in the person of county agents, extension specialists, company representatives, and research workers. Emphasis could now be placed on the disease and its control, not exclusively on the fungus and its taxonomy.

Not only could Thaxter and his few contemporaries now earn a living at plant pathology, so could some 1600 others, who now comprise the membership of the American Phytopathological Society.

In 1888 Thaxter was hired by The Connecticut Agricultural Experiment Station and was thus thrust into all the new problems of plant protection. He published his "lotion bulletin" on Bordeaux in 1890 and the Station has been having "fun with fungicides" ever since.

Thaxter's fun with fungicides was pungent, sometimes bitter. He called plant pathologists, "squirt gun botanists," an opprobrious term that has been difficult to live down. Nevertheless, it has been lived down. Plant pathologists have done much research in the basic sciences that underlie the principles of protection. It is my pleasure to record some of it herein.

The orthodox paper on mechanisms of action takes the point of view of the compound. What, for example, do dithiocarbamates do to fungi? Since I am unorthodox, I prefer to take the fungus-eye view. What significant functions of the fungus are affected by dithiocarbamates?

## II. Methods to Measure Mechanisms

If we are going to do things to fungi with fungicides, we must arrange for the fungus to respond in some way that we can comprehend. Fungi are simple organisms. Their responses are, therefore, simple. They can grow larger; they can take up oxygen or give off $CO_2$; they can reproduce; their spores can germinate. These are the common responses that are used to assay the action of fungicides. Fungi can do a few other things that we can measure, but the measurement becomes pretty difficult. They can probably synthesize guanine, for example, and hundreds of other compounds, but measuring the results of such syntheses becomes a bit tedious. More of such measurements will have to be made, however, as we delve deeper into mechanisms of fungitoxicity.

And speaking of fungitoxicity, we shall use that term rather than fungicidal value. The former implies deleterious action of all ilks. The latter is limited to deleterious action that results in death.

Three manipulative maneuvers are in common usage for measuring toxicity—the poisoned food technique, the spore germination technique, and the manometric technique. Animal physiologists use a technique of macerating the tissues to obtain intracellular bodies for manipulation. These techniques are just now entering the field of fungicides, as witness the recent papers by Owens (54). The data discussed here have all come, however, from the first three techniques named.

In all three of the techniques, it is advisable to investigate a range of doses of the test compound. In the first case the candidate material is incorporated into the food supply of a growing fungus. There the compound may interfere with any one of the hundreds or thousands of reactions that are carried on by the intact fungus.

In the second instance the spore, which generally carries all or almost all of its rations, germinates in the presence of the test material. The number of avenues of attack by the compound is reduced and the problem of appraisal is, thus, somewhat simplified.

In the third instance, the gas exchange is measured in the presence of the test material. Gas exchange is related to still fewer mechanisms and a still simpler problem faces the investigator.

In the fourth case of fungus macerates, the technique appraises a still simpler set of systems.

Once the responses are quantitatively appraised, they may be related graphically to the doses used (31). In so doing a dosage-response (DR) curve is generated which has both position and slope. Position ($ED_{50}$ = effective dose for 50% response) measures potency; slope measures mechanism. If compound A is more potent than B, the $ED_{50}$ is smaller. This may be because it has a smaller particle, because it permeates the cell more easily, or simply because it is a more effective fungicide.

If the slope of *A* is steeper or flatter than *B*, one can be reasonably sure that the two compounds affect different mechanisms. If the slopes are identical, one cannot be sure whether the mechanisms differ or not.

## III. Problems of Penetrating the Protoplast

The living protoplast of the fungus, like the living protoplast of all cells, is surrounded by a chemical barrier. This is the famed semipermeable membrane. If you want to have scientific fun, just try to drive an unusual molecule through this barrier. Your success as a discoverer of a new fungicide will depend in no small measure on the degree to which you can do this.

This is one of the features of fungicide research that has been neglected. Those whose business it has been to devise new fungicides have virtually ignored it.

They have usually used a "screening technique" sometimes described as the "Edisonian" technique—try everything once.

Knowing that a fungus is an aqueous chemist, they have tried another tack. They have attached a reactive group to some handy nucleus that was already being merchandized for the rubber business or whatnot. Sometimes this has achieved spectacular results as, for example, in the recent development of captan. Kittleson (39) tells me in conversation that he thought it would be fun to try to react a secondary amine with perchloromethylmercaptan. The reaction did go and Kittleson discovered what he published as a "new class of fungicides." Captan (I) is, of course, the best known example.

In more cases the compound that is screened out is subjected to intensive research with various substituents. Many times the successful substituent turns out to be a long hydrocarbon tail or an aromatic ring or two.

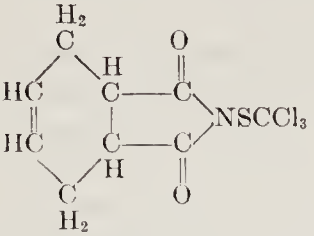
An excellent example of this is gliodin (II), first published by Wellman and McCallan (70). They were cooperating with Carbide and Carbon Chemicals Company. Carbide and Carbon is a large supplier of ethylene diamine. Naturally, they tested derivatives of ethylene diamine. The initially screened compound was not identified in their paper, but they arrived at the commercially successful derivative, 2-heptadecyl-2-imidazoline (II), by running through the range of hydrocarbon chains in the 2-position, finally settling on the 17-carbon tail. The decision to make this derivative was probably dictated also in part by the relative cheapness of the stearic acid intermediate.

Soon after Wellman and McCallan (70) published on gliodin Rader *et al.* (56) published on the toxicity of tetrahydropyrimidine substituted also with 17-carbons.

It should be noted that the improvement in performance was derived in both these cases from a long hydrocarbon tail, a derivative that could hardly be dreamed of as improving reactivity in an aqueous medium. In fact the 17-carbon tail is so long that the compound is rapidly squeezed out of solution onto the glass of a beaker or onto whatever solid object is present, including the spore.

What all these researchers succeeded in doing was to drive a toxicant through the semipermeable membrane. The semipermeable membrane in the spores of my fungus, *Stemphylium sarcinaeforme*, will exclude 2-methyl-2-imidazoline and the spore will not be poisoned by it. The semipermeable membrane of the spore cannot exclude 2-heptadecyl-2-imidazoline. The compound permeates the spore and kills it.

This conclusion, which was derived by Rich and Horsfall (60), led us to investigate another compound which had long puzzled us. Barratt and Horsfall (6) had some years earlier shown that ethylenethiourea (III) occurs in the debris when disodium ethylenebisdithiocarbamate (IV) spontaneously degrades.



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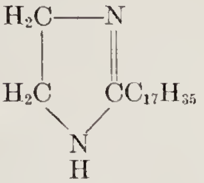

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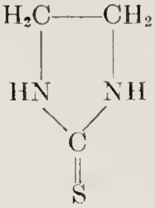

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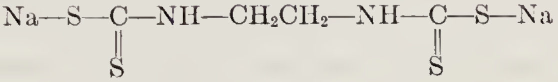


$$Na{-}S{-}\underset{\underset{S}{\|}}{C}{-}NH{-}CH_2CH_2{-}NH{-}\underset{\underset{S}{\|}}{C}{-}S{-}Na$$

(IV)

It had long seemed to us that this compound should be fungitoxic, but it was not. This was difficult to understand in the light of data that we had published (32) on numerous other heterocyclic nitrogen compounds. We concluded that in general such structures are fungitoxic. The best explanation for the inactivity of ethylenethiourea was that it was incapable of passing through the semipermeable membrane. We decided that it needed a hydrocarbon tail.

At our request, Dr. C. P. Lo of the Rohm and Haas Company attached an 8-carbon hydrocarbon tail to one of the nitrogen atoms. To our distinct pleasure the resulting compound turned out to be highly fungitoxic (61). Seldom have we succeeded in converting an inactive toxiphore to an active compound. The result lends hope, however, that other heterocyclic nuclei can be converted to active structures by means of hydrocarbon substituents.

The explanation for the action of the hydrocarbon tail may be derived from the generally accepted structure of the semipermeable membrane itself. Davson and Danielli (12) present a beautifully well integrated argument for the nature of the membrane.

According to their summary of the matter, the semipermeable membrane is analogous to a sandwich. The slices of bread are protein layers on either side of a fatty middle. A fungus cell may be considered schematically as a bag of water immersed in water. In other words the cell membrane is wetted both inside and out.

It is well known from milk that when fat and protein are suspended together in an aqueous medium the fat clings to the lipid groupings of the protein and the water clings to the polar moieties. Since a protein molecule is thus partially surface active, it, in effect, surrounds the fat and forms globules.

Similarly, in the aqueous system of the fungus cell, the protein comes to surround the fat and shield the fat from the water. Since water is on both sides, the fat comes to occupy the center of the sandwich.

Now when a fungicidal (sic) molecule is applied to such a protein-fat membrane, what happens is governed by the interaction of the two structural units. If the molecule is too water loving (hydrophilic), it tends to be excluded by the fatty filling in the middle of the membrane. If the molecule is too water hating (hydrophobic), it is excluded by the water soluble groups on the outer face of the protein layer.

Clearly there must be a balance between fat solubility and water solubility. The ratio between fat solubility and water solubility is often called partition coefficient. This means that if the test compound is dissolved in a fat-water mixture, it will partition itself between the two in proportion to its relative solubility.

Rich and Horsfall (60) have shown that the fungitoxicity of nitrosopyrazole derivatives is proportional to fat/water partition coefficient. This, of course, is no new concept. Bateman (7) as far back as 1922 had investigated this possibility. One of the problems, however, that pestered Bateman was that, in a series of homologues, activity rises to a peak with partition coefficient and then declines. He could not understand the decline. The decline is pretty clearly due to the fact that if the compound is too fat soluble, then it cannot pass through the hydrophilic outer layers of the protein. Macy (43) has recently compiled an extensive list of partition coefficients. It would be interesting to relate Macy's values to the fungitoxic values for the same compounds.

The fat-solubilizing substituents that improve permeation are called shaped charges by Rich and Horsfall (60). This name has been applied because their activity is analogous to shaped charges in artillery shells. Ballistic experts concern themselves with driving a shell through an armored barrier. If the explosive exerts force in all directions, the penetrating power of the shell is small. If, however, the charge is confined into a certain shape by the internal dimensions of the shell, its major explosive force will be directed toward the front end of the shell, not in all directions. Thus, with the same amount of explosive, one shell will penetrate a piece of armor plate. Another will not. And one fungicide will penetrate the semipermeable barrier. Another will not. One has a shaped charge. The other has not.

Let us return to Kittleson's (39) killer, captan (I). Kittleson assumed that the —S—C(Cl)$_3$ moiety in captan is the toxiphore. We shall give data below to suggest that the tetrahydrophthalimidine is the toxiphore and we suggest here that the —C(Cl)$_3$ is a shaped charge. It is certainly a strongly lipophilic group and almost certainly serves as a shaped charge. It is true that the —C(Cl)$_3$ group is also strongly electronegative. It may well withdraw electrons from the sulfur and activate it. If so, and to that extent, it contributes twice to the toxiphore. At present, our feeling (33) is that it serves chiefly as a shaped charge and, hence, that Kittleson had a "new type of shaped charge" not a "new type of fungicide" in the strict sense.

## IV. Rupture of the Structure of the Cell

In this section, I shall have fun being unorthodox again. Ordinarily we consider the action of fungicides (and drugs too) as due to disrupting the chemical symphony in the cell. We generally understand that the chemical reactions proceed smoothly to their conclusion in the production of fungus stuff. If, however, we introduce a foreign molecule into the system, the balance goes out of kilter and the organism dies. In short, it is poisoned.

At the cellular level, however, it is difficult to distinguish between poisoning in the usual sense and some other effects. One of these other effects is wounding and another is starvation. In this section we shall try to equate wounding and poisoning. We shall suggest that some compounds may deliver a mortal wound to the cell, so that, for example, it bleeds to death rather than is poisoned.

We can best illustrate this principle for our purposes here by considering the effect of compounds on the semipermeable membrane. In the last section we considered the effect of the membrane on the activities of the compound. Here let us consider the effect of the compounds on the activities of the membrane.

As we mentioned above, the membrane is bounded on either side by a layer of protein. It is the protein which apparently gives integrity to the membrane. It seems to be responsible for keeping the membrane intact—in short for maintaining its selective quality.

Before proceeding to the problem of wounding the membrane, we should consider the nature of the protein layer that binds the membrane. Frey-Wyssling (18) in his beautiful book on the microstructure of protoplasm points out that protein layers are made up of long protein molecules held together by lateral linkages of several types: (a) homopolar lipid bonds (hydrocarbon), (b) homopolar dipole acid bonds (COOH and OH), (c) homopolar dipole basic bonds ($NH_2$), heteropolar valence bonds (esters and amides), and homopolar valence bonds (ethers and disulfides).

The semipermeable membrane can keep materials inside the cell or it can keep them out. In that sense, then, a wound in the membrane might act under some circumstances to permit an excessive amount of material to enter the cell. In other cases, it might allow an excessive amount to escape.

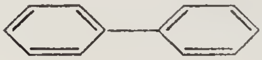
The experimenter's problem is to design a test to ascertain whether wounding is or is not so acting.

I think we can bring data to bear on the first three types. That is, there appear to be reasonably good data for disrupting with fungicides the lipid bonds, the acidic bonds, and the basic bonds. These data will be concerned with ingoing and outgoing materials.

The data we shall use are literature data, none of which have been used hitherto to test this hypothesis. The data are from two types of experiments: (A) The compound and fungus cells are placed together in an aqueous medium. (B) They are placed together in a dry environment.

Under the moist conditions excessive inbound motion of materials (probably mostly water) would be evidenced by explosion of cells or at least by excessive bloating. An excessive outbound motion would be evidenced by excessive amounts of metabolites in the liquid leaving the cells.

No cell has been observed to explode under dry conditions. Presumably under these conditions water would escape excessively and the cell would die of desiccation. Available work on dry cells has been done with powdery mildew on the surface of the host.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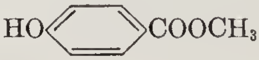


(VI)

Considering first the homopolar lipid bond, we ought to be able to dissolve it in fat solvents. Diphenyl (V) is a commercial fungicide that is a reasonably good fat solvent. Ramsey *et al.* (57) have reported that it causes *Botrytis* spores in water to burst. The inbound movement of water (presumably) has been too much for the spore wall to stand. Vincent (69) has shown that methyl-*p*-hydroxybenzoate (VI) will cause certain fungus spores to swell to grotesque bloated forms.

So far as outbound water is concerned, Barker and Lees (5) several years ago showed that kerosene emulsion would bring on the death of powdery mildew hyphae and conidia. Somewhat later (45) glycerides were found to be effective and still later (46) it was found that a wide variety of commercial solvents display the ability to kill powdery mildew. This presumably results in all cases from excessive water loss. The powdery mildew fungus residing outside its host is particularly vulnerable to water loss.

Perhaps the best known metabolite to leak from fungus cells is penicillin. Although they could not explain it, Goldschmidt and Koffler (23) were able to double the yields of penicillin by adding a glyceride or a mineral oil defoamer to the fermenters. I suggest that these fat solvents wounded the membrane slightly and reduced its ability to hold the penicillin in.

In considering the nature of the dipole acidic bonds, one might expect that cations would combine with and weaken that linkage. Such appears to be the case.

Several years ago Kunkel (40) reported that spores treated with metal cations will lose metabolites to the surrounding medium. Goodwin *et al.* (24) discovered that alkalis cause zoospores of the hop downy mildew fungus to explode. Gattani (22) observed a similar result for rust urediospores. We may assume that too much water enters through the wound made at the acid linkage.

So far as outgoing water is concerned Yarwood (75) has shown that alkalis are good to kill powdery mildew. They probably allow the spore to lose water irreversibly.

Cationic detergents also should affect the acidic bonds. In this connection Hotchkiss (36) has shown that such compounds encourage the leakage of nitrogenous and phosphoric constituents from bacterial cells, and Yarwood (75) has shown that they kill powdery mildew.

Insofar as the homopolar basic linkages are concerned, one would expect that anion active compounds would disrupt these. Goodwin *et al.* (25) showed that ordinary sodium soaps cause zoospores of hop downy mildew to explode and Görnitz and Harnack (26) found that sulfonated terpenic alcohols cause miscellaneous spores to explode. Yarwood (75) reported that anion active surfactants destroy powdery mildew conidia, and finally Hotchkiss (36) found that they also cause leakage of nitrogenous and phosphoric metabolites from bacterial cells.

Thus we find that we can wound the semipermeable membrane with chemicals that have the ability to attack at least some of the lateral linkages in the proteins. Such wounds may on some occasions allow too much water to enter and the cell explodes. In other cases they allow too much water to escape and the cell dies by desiccation.

## V. Effect on Metabolism of the Cell

The fungus, like essentially all other organisms, must provide itself with food and shelter. We have discussed its shelter problems above. Here we shall discuss its food supply and how fungicides upset it.

A fungus, unlike its brethren in the plant kingdom has no chlorophyll and thus must feed on organic matter living or dead. In this sense it is more closely allied to animals than to plants. In general, much of its food must be pre-elaborated. It is usually dependent upon outside sources for vitamins, for example.

The literature provides some reasonably respectable data on the effect of fungicides on the food fancies of the fungi. The literature is a little weak in information on intake. Wood rotting fungi require glucose which they obtain from cellulose. They excrete an enzyme, cellulase, which hydrolyzes the cellulose to glucose. Finholt *et al.* (15) have clearly shown that their fungus will be killed by certain aliphatic amines, *provided* the fungus first is forced to subsist on cellulose. These compounds are bland if glucose is supplied. In other words, the predigestion of cellulose to glucose is inhibited. It would be interesting to try Finholt's compounds on termites. Gale and Mitchell (20) have similarly shown that triphenylmethane dyes inhibit glutamic acid assimilation by bacteria.

One of the most popular, certainly one of the most controversial and thus one of the most interesting, areas in nutrition is metal nutrition. Fungi, like all organisms, must have metals to live, certainly copper, iron, calcium, zinc, manganese, potassium, and molybdenum (17).

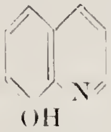
Zentmyer (77 and 78) some years ago in our laboratory suggested that oxine (8-hydroxyquinoline) (VII) probably owes its toxicity to its ability to rob the fungus of its metals. Hence, chelation should be a potent mechanism of fungitoxicity. This hypothesis has had its strong supporters and strong opponents. We have gleefully joined the fray. Whether we ever settle the matter is, perhaps, insignificant. We are sure to learn a lot about fungi in the process of investigating the matter.

The battle swirls around the problem of oxine. Looking back on it, one might wish that Zentmyer had chosen a different experimental material. His paper pointed the spotlight on oxine. Now we know that oxine exerts other types of toxicity than chelation. It surely chelates metals and presumably (we think) robs the fungus of its metals. On the other hand, it also contains a heterocyclic moiety (quinoline) which, according to our studies (32) probably is toxic in its own right, especially if it can be given the proper shaped charge to penetrate the cell.

Oxine shows a bimodal dosage-response curve (32). We think that this derives from its two types of toxicity, quinoline poisoning and chelation. Whether this is true or not, the double curve makes it extremely liable to experimental mishaps. One never knows for sure which peak he is dealing with. When he manipulates the molecule and reduces toxicity or increases toxicity, he hardly knows for sure which peak he is climbing or descending.

Perhaps it would be simpler to use simpler chelating molecules first to set up some guideposts. One line of reasoning that helps to convince us of the importance of chelation as a fungitoxic mechanism, is that we can induce symptoms of metal deficiency by growing the fungus on medium plus chelator. Spores of *Aspergillus niger* are black. The color is copper dependent as Bortels (9) has shown. Rich and Horsfall (59) have shown that the fungus can be forced to produce bleached spores, if dimethylglyoxime or hydrogen sulfide is present in the medium. They later (35) showed that numerous chelators bleach hyphae of *Monilinia.* Van Raalte (55) repeated these results with dithiocarbamates. He even went further. He reversed the effect by adding stoichiometric amounts of extra copper to balance the chelator.

This business of reversal has been a barrier to acceptance of the chelation hypothesis mostly because of researches on oxine. Van Raalte (55) reversed his dithiocarbamate. Horsfall (31) has shown that salicylaldoxime (VIII), mercaptobenzothiazole (IX), and pyridine-3-thiol (X) can be reversed by metals. But you can seldom reverse oxine. Often its toxicity can be reduced, seldom completely nullified. We feel that the reason is simple. Oxine has two modes of action. The best you can do is reverse one of them with metal.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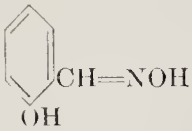
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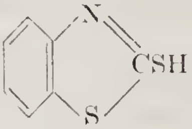
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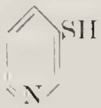

(X)

The doubting Thomases really get fire in their eyes when they cite the fact that the copper chelate of oxine is far more toxic than either the copper or the oxine. This is worsened by the demonstration in our laboratory that several copper chelates (such as copper acetonylacetonate (XI)) at sub-lethal concentrations will increase sporulation of *Monilina fructicola* presumably by providing it with copper.

This problem is eased somewhat by the counter fact that the copper chelate of salicylaldoxime is far, far less toxic than either ingredient (31). Any working hypothesis must encompass both sets of facts.

My own feeling is that the explanation of the differences must lie either in differential permeability of the two chelates or differential dissociation once the compounds are inside the cell. Most of the discussion here must deal with copper oxinate since it is the nonconformist.

Anderson and Swaby (2) and Block (8) have shown that copper oxinate is more fat soluble than either ingredient and presumably, therefore (see above), permeates more readily. The chelate has such a very high stability constant that many persons feel that it does not dissociate once inside the cell. If so, the toxicity must be due to the heterocyclic quinoline nucleus. There is no comparable nucleus for the copper of salicylaldoxime. On the other hand the copper oxinate must dissociate because Greathouse *et al.* (27) developed a strain of *Aspergillus niger* that is resistant to copper ozinate. It is resistant, however, only to the copper moiety not to the oxine moiety.

If copper oxinate dissociates within the cell, it must do so because some natural chelator within the cell seizes the copper. This could well be histidine, since Albert (1) showed that copper histidinate has a higher stability constant than copper oxinate and Zentmyer and Rich (79) have just shown that histidine will reverse the toxicity of copper oxinate.

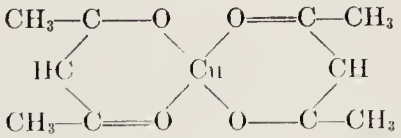
Thus the copper poisons the histidine system and the oxine residue either poisons by chelating other metals below copper in the Mellor-Maley (49) series or the quinoline moiety is the active grouping. In either case the extra toxicity seems to be due to the *extra* permeation induced by forming a superior "shaped charge" when the chelate is made. In this manner both toxic moieties gain entrance to the cell and both are toxic inside.

Presumably the copper chelate of salicylaldoxime either does not penetrate well, or it is not easily dissociated once inside.

In connection with the latter possibility, Müller and Biedermann (50) have shown that treatment with ethylenediaminetetraacetic acid will resuscitate spores of *Alternaria tenuis* that have recently been treated with copper salts, and Yoder (76) was able to send cysteine into the cells of *Monilinia fructicola* and nullify the toxic effects of copper.

The research on chelation as a toxic mechanism will surely go on and contribute additional fun with fungicides.

The other field of metabolism that is active just now is that of antimetabolites. Woods (72), the bacteriologist, broke down the fence into this field in 1940 when he suggested that the wonder drug, sulfanilamide (XII), owed its activity to its competition with *p*-aminobenzoic acid (XIII), a normal metabolite.



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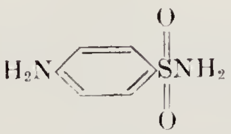
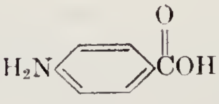


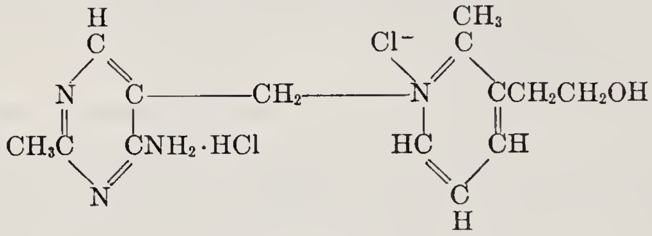
(XII)



(XIII)

This was a new idea in toxicity. In effect Woods was saying that the old theatrical dodge of mistaken identity works also in the field of toxicity. As can be seen from their structural formulas, these two compounds do look alike. Both are aniline derivatives with *para* substitutions, one a carboxylic acid group and the other a sulfonic acid group. Chemists often say that carboxylic acids and sulfonic acids act similarly.

Woods says in effect that bacteria cannot tell the difference. They reach for sulfanilamide under the impression (if you will excuse the expression) that it is *p*-aminobenzoic acid. When they incorporate this into their tissues, it does not work well enough and they die.



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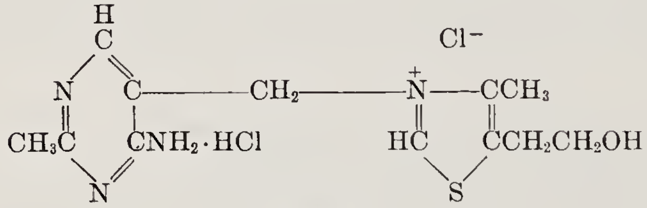
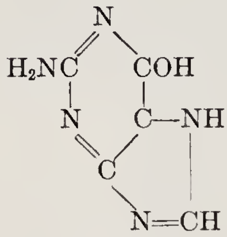
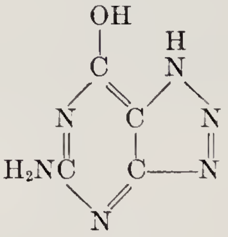


(XV)



(XVI)



(XVII)

This new idea was meat for Robbins' grinder (62). He had long been interested in the vitamin requirements for fungi and he soon showed that pyrithiamine (XIV) (Neopyrithiamine) is a competitive inhibitor for thiamine (XV). The fungus cannot tell them apart until too late. Pyrithiamine differs from thiamine only in that a pyridine ring is substituted for a thiazole ring. It is what is known as an isostere. The —CH=CH— of the benzene ring substitutes for the —S— of the thiazole ring and occupies the same space, but it does not perform the same physiological function.

Quite recently, West and Wolf (71) have shown that the 2-heptadecyl-2-imidazoline (II) of Wellman and McCallan (70) is a competitive analogue of guanine (XVI). Fries and Panders (19) have shown that guanine may be antagonized competitively also by 5-amino-7-hydroxytriazolopyrimidine (XVII) (guanazolo) for *Ophiostoma multiannulata.*

It is interesting to note that the imidazoline compound is toxic to both our test organisms, *Monilinia fructicola* and *Stemphylium sarcinaeforme,* but the triazolopyrimidine compound is not. Presumably the latter compound does not permeate into our organisms because I cannot believe that they have no guanine requirement. The latter compound has a large number of hydrophilic groupings. These might well exclude it from many organisms.

Once Woods broke open the fence into this field of antimetabolites, researchers have swarmed through the gap like the settlers into Oklahoma when the fences were dropped in 1889.

For a long time we were quite content to assume that heavy metals killed protoplasm by reacting with the amino acids of the proteins. Recently MacLeod and Snell (42) have tried to put the action of heavy metals also under the comfortable umbrella of antimetabolites. This, I admit, is not unreasonable. They say that "certain metallic ions that inhibit growth may be looked upon as structural analogues of other metallic ions which are essential. . . ."

And now even the classic old fungicide, elemental sulfur, is scrouging for space under the same umbrella. Ever since Rey-Pailhade (58) in 1888, we have known that sulfur is reduced to hydrogen sulfide by fungi. Beginning with Barker (4) and Marsh (44) we have known that hydrogen sulfide is toxic to fungi. How simple? The sulfur is reduced to hydrogen sulfide by the fungus, and the hydrogen sulfide poisons it. Suicide again.

It was left to nonfungicide experts to set this in proper perspective. Nord, a biochemist, has long been concerned with respiration of *Fusariums.* Sciarini and Nord (63) showed rather plainly that the fungus confuses oxygen and sulfur. After all, these elements are close neighbors in the periodic table. The fungus reduces the sulfur to $H_2S$ and not the oxygen to $H_2O$. In the process the sulfur gets involved in respiration instead of oxygen; the cell cannot stand the disruption, and it dies.

This is a classic case of deriving an almost diametrically opposite conclusion from the same data. Sciarini and Nord conclude that the cell is killed by *producing* hydrogen sulfide, not by the hydrogen sulfide itself.

This explains a lot of anomalies. In practice powdery mildew fungi are the prize targets for sulfur. It is very toxic to them. And yet we have known since Eyre and Salmon (14) in 1916 that these fungi are not sensitive to hydrogen sulfide.

Sulfur is a reasonably lipophilic compound and presumably permeates reasonably well. The powdery mildew fungi contain more fat than usual among fungi as Yarwood (74) has found. They are presumably easily permeated by sulfur.

Our two test fungi differ in their permeability. *S. sarcinaeforme* is permeable to the more hydrophilic types of molecules, and *M. fructicola* to the lipophilic ones. One might expect then that the latter would be more sensitive than the former to sulfur which is a lipophilic substance. And this is well known.

A few other examples of interference with metabolism have been established. Respiration and the enzymes of respiration have been investigated by Owens (52 and 53) and by Sisler and Cox (66). The effect of compounds on respiration and the utilization of glucose in respiration are presumably effects on the enzymes involved.

Enzymes, being proteins, contain various reactive groups. Among these are carboxyl, amino, hydroxyl, and sulfhydryl groups. Reagents that react with these should inhibit some or all of the enzyme activity.

Metals inhibit respiration presumably by forming chelates with the amino and carboxyl groups. Quinones, prominent fungicides, probably act by forming addition compounds on the $\beta$-carbon with amino, hydroxyl, and sulfhydryl groups as suggested by Hochstein and Cox (29).

Dithiocarbamates, according to van der Kerk *et al.* (38) have the faculty of combining with the sulfhydryl groups although in our laboratory we believe that they also interfere with terminal oxidases by chelating the metals in the coenzymes as well.

The dinitro containing fungicides seem to produce their effects by inhibiting phosphorylation reaction (65).

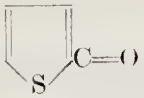
One of the interesting physiological responses not yet adequately explained is Emerson's (13) discovery that furfural and furfuryl alcohol will reverse the dormancy of *Neurospora* ascospores. Sussman (68) has shown that 5-membered heterocyclic compounds like thiophene, furan, and pyrrole are active. A ketone in the 2-position seems to improve activity. This is interesting to us because Dr. Rich and I have found that a 2-keto thiophene (XVIIa) (35) will greatly reduce sporulation of *Monilinia fructicola* but that thiophene (XVIII) itself will not.

## VI. Effects on Reproduction

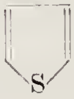
A noteworthy feature of fungicides is that usually they are aimed at killing spores, seldom or never at reducing number of spores. In our laboratory we have been investigating for some years the effect of compounds on asexual sporulation of *Monilinia fructicola* and a few other fungi. We hope some day to develop a compound for plant disease control, the action of which is based on the inhibition of sporulation.

It is probable that some diseases are or have been controlled by means of antisporulants. Some years ago we suggested (30) that the control of tobacco downy mildew by benzene and *p*-dichlorobenzene and the control of *Botrytis* on lettuce by pentachloronitrobenzene (XIX) is due to the prevention of sporulation.

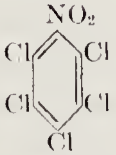
Our first success in preventing sporulation came with the use of chelating agents. Bortels (9) showed that *Aspergillus niger* needs metals to grow well and sporulate freely. My colleague, Dr. Rich, and I (59 and 35) set out to reduce sporulation by treating the growing fungus with a chelating reagent.



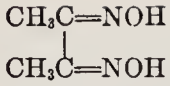
(XVIIa)



(XVIII)

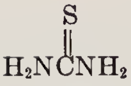


(XIX)



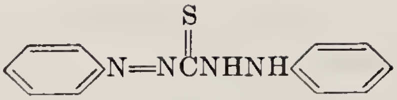
$$\begin{matrix} CH_3C{=}NOH \\ | \\ CH_3C{=}NOH \end{matrix}$$

(XX)

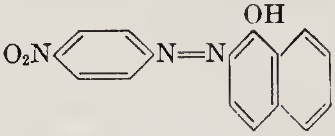


$$\overset{S}{\overset{\|}{H_2NCNH_2}}$$

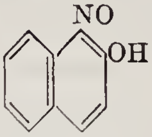
(XXI)



(XXII)



(XXIII)



(XXIV)

The following are some of the chelators that have been effective: dimethylglyoxime (XX), thiourea (XXI), diphenylthiocarbazone (XXII), *p*-nitrobenzene azo-α-naphthol (XXIII), 1-nitroso-2-naphthol (XXIV), potassium ethylxanthate (XXV), and thioglycolic acid (XXVI). Similarly, Fleury (16) was able to reduce the sporulation of *Aspergillus niger* with thiourea, a metal-binding agent. Yarwood (73) years ago succeeded in preventing sporulation of *Peronspora destructor* on onions with lime sulfur. This probably resulted from the binding of the metals by the hydrogen sulfide, which is liberated by fresh lime sulfur.

One might expect that mitotic inhibitors in the sense of Östergren (51) might inhibit sporulation by inhibiting the formation of the necessary new nuclei. We have found (35) that the carbamate relatives of urethane, especially the sulfur analogues, do reduce sporulation. Also numerous hydrocarbons and chlorinated hydrocarbons, that are known to affect mitosis, act as sporulation inhibitors.

As we have shown (34), ketones and carboxylic acids in general are strong inhibitors of sporulation. While investigating this problem, we discovered (35) that α,β-unsaturated ketones in general stimulate sporulation. We were pleased to find some compounds to stimulate sporulation because we need both ends of the scale to study the matter.

It soon became apparent in studying the α,β-unsaturated ketones that if the β-carbon is blocked or sterically hindered, the compound fails to stimulate sporulation.

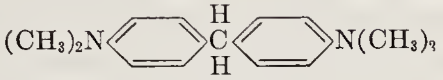
From this we were led to infer that perhaps the fungus liberates into the medium a sporulation inhibitor. If so the β-carbon of the α,β-unsaturated ketone must form an addition compound with the inhibitor and thus prevent its action. Perhaps it is pointless even to speculate on the nature of this unknown inhibitor. β-Carbons that are activated by a ketone group do combine with amino and sulfhydryl compounds, however.

$C_2H_5O\overset{\overset{S}{\|}}{C}SK$

(XXV)

$HSCH_2COOH$

(XXVI)



(XXVII)

Another interesting group of sporulation inhibitors are those that are relatives of bis(4-dimethylaminophenyl)methane (XXVII) (Arnold's base). Actually, this compound itself is only weakly active, but while exploring in its chemical vicinity, we discovered two quite active compounds, acridine red and rhodamine B. These compounds also enhance sporulation of *Aspergillus niger*, *Graphium ulmi*, and *Alternaria oleraceae.*

What characterizes these compounds is that they all contain two dimethylamino groups that are rather widely spaced. A very rough estimate suggests that activity increases with their distance apart at least up to 10 A. Although there is not a shred of evidence for it, one toys with the hunch that these compounds may influence somehow the coils of the molecules of nucleic acid.

## VII. The Fungus Fights Back

For every reaction, say the chemists and physicists, there is an equal and opposite reaction. For every effect of a compound on a fungus, cell, there must be an effect of the fungus on the compound.

My estimate is that *Phytophthora infestans*, the potato blight fungus is much more resistant to copper along the Atlantic seaboard than it was in 1886 when Bordeaux mixture was first introduced to America.

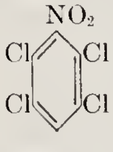
Copper, as Bordeaux mixture, is a well-known fungicide. Here the copper acts as a cation and its activity almost surely is to combine with the acidic groups on the protein as long ago suggested by Loew (41). Since the exterior of the semipermeable membrane is presumably protein, we can reasonably assume that the first copper at least binds on to these proteins. In so doing it pushes aside the hydrogen ions.

In general the fungi that are naturally resistant to copper toxicity and also those that have been bred resistant have one feature in common. They are heavy excretors of hydrogen ions (28,67). They run the pH of the medium down. It seems probable indeed that these organisms fight back against copper poisoning by producing an overwhelming supply of hydrogen ions that tend to keep the copper away from the surface proteins. Scott's (64) data show clearly that copper is less toxic at low pH than at high.

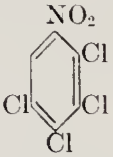
We do not know how *Phytophthora infestans* fights back against copper, but Arakatsu (3) shows that a copper resistant yeast synthesizes very much more glutamic and aspartic acid than the nonresistant strain. Possibly these amino acids chelate and inactivate the copper.

Presumably other heavy metal cations affect the fungus by a mechanism similar to that exerted by copper. Noteworthy, therefore, is Jurkowska's paper (37) that a strain of *Aspergillus niger* bred for resistance to copper was likewise resistant to zinc.

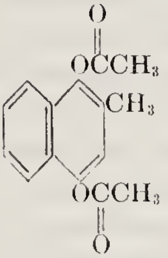
On the other hand one would expect that a fungus resistant to a metal might not be resistant to an organic fungicide. Gattani (21) has shown just that a strain of *Alternaria* sp. resistant to an organic mercury compound was not resistant to tetramethylthiuram disulfide.



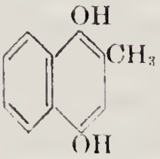
(XXVIII)



(XXIX)



(XXX)



(XXXI)

The specificity may be even narrower. McKee (48) has developed a strain of *Fusarium caeruleum* which is resistant to 2,3,5,6-tetrachloronitrobenzene (XXVIII), but Brook (10) has found in turn that it is not resistant to the 2,3,4,5-isomer (XXIX).

Occasionally, when a fungus attacks a foreign compound within its body, it worsens the matter.

Some years ago (30) we noticed that 2-methyl-1,4-diacetoxynaphthalene (XXX) is not toxic to *Monilinia fructicola,* but is toxic to *Stemphylium sarcinaeforme.* Our memory was jogged a few years later when Byrde and Woodcock (11) noticed a similar result for the 2,3-dichloro analogue. Upon investigation, they demonstrated rather clearly that the sensitive fungus hydrolyzes the ester linkage and forms the dihydroxy analogue (XXXI) which is toxic.

In this case the fungus commits suicide in the presence of 1,4-diacetoxynaphthalene. The same situation probably obtains also for Bordeaux mixture. It is impossible to extract with water enough copper from Bordeaux mixture to kill a fungus, but McCallan and Wilcoxon (47) showed that excretion from the spores will dissolve out a lethal dose of copper from Bordeaux mixture. Thus, the spores poison themselves.

## VIII. Summary

And thus we come to the summary. It is fun to do research with fungi and fungicides. They can be made to "sit up and beg" and the techniques are relatively simple. One of the really fascinating problems is permeation and this is usually solved by searching for the proper balance of fat and water solubility. At the same time we find that fungicides may wound the semipermeable membrane so that substances rush in too violently or leak out too easily, and thus the fungus dies. Thus, in this case, poisoning is equivalent to wounding.

In other cases poisoning is equivalent to starvation. Fungicides may combine directly with foodstuffs as, for example, metals or phenols with proteins. They may also compete with normal metabolites. If they closely resemble a metabolite, the fungus may confuse the two and die. This may even apply to simple fungicides like sulfur which acts as an antimetabolite for oxygen or poisonous metals which may act as antimetabolites for essential metals.

Some synthetic compounds such as chelators and mitotic poisons inhibit sporulation of fungi and $\alpha,\beta$-unsaturated ketones enhance it, presumably by forming addition compounds with some naturally occurring inhibitor of sporulation.

Fungi fight back against fungicides more often than we realize.

For example, they excrete large masses of hydrogen ions and thus prevent metal poisoning.

The fight with the fungi goes on.

## References

1. Albert, A., Quantitative studies of naturally occurring substances for trace metals, 1. Amino acids having only two ionizing groups. *Biochem. J., 47,* 531 (1950).
2. Anderson, B. I., and Swaby, R. J., Factors influencing the fungistatic action of 8-hydroxyquinoline (oxine) and its metallic complexes. *Australian J. Sci. Research Ser. B, 4,* 275 (1951).
3. Arakatsu, Y., Yanagishima, N., Minagawa, T., Naiki, N., and Ashida, J., Studies on the adaptation of yeast to copper, X. Effect of nutrients and inhibitors on the growth and pigmentation in the presence of copper. *Mem. Coll. Sci, Univ. Kyoto Ser. B, 21,* 97 (1954).
4. Barker, B. T. P., Investigations on the fungicidal action of sulphur, IV. Third progress report, 1930, *Ann. Rept. Agr. and Hort. Research Sta., Long Ashton, Bristol, 1929,* 130.
5. Barker, B. T. P., and Lees, A. H., Kerosene emulsion against gooseberry mildew. *Ann. Rept. Agr. and Hort. Research Sta., Long Ashton, Bristol, 1914,* 73.
6. Barratt, R. W., and Horsfall, J. G., Fungicidal action of metallic alkyl bisdithiocarbamates. *Conn. Agr. Expt. Station Bull. 508* (1947).
7. Bateman, E., Theory on the mechanism of protection of wood by preservatives, III. Experimental proof of the theory by means of the toxicity and solubility partition of a number of tar acids. *Proc. Am. Wood-Preservers' Assoc., 18,* 70 (1922).
8. Block, S. S., Fungitoxicity of the 8-quinolinols. *J. Agr. Food Chem., 3,* 229, (1955).
9. Bortels, H., Über die Bedeutung von Eisen, Zink, und Kupfer für Microorganismen. *Biochem. Z., 182,* 301 (1927).
10. Brook, M., Differences in the biological activity of 2,3,5,6-tetrachloronitrobenzene and its isomers. *Nature, 170,* 1022 (1952).
11. Byrde, R. J. W., and Woodcock, D., Fungicidal activity and chemical constitution, II. Compounds related to 2,3-dichloro-1,4-naphthaquinone. *Ann. Appl. Biol., 40,* 675 (1953).
12. Davson, H., and Danielli, J. F., *The Permeability of Natural Membranes,* Cambridge Univ. Press, Macmillan, New York, 1943.
13. Emerson, M. R., Chemical activation of ascospore germination in *Neurospora crassa. J. Bacteriol., 55,* 327 (1948).
14. Eyre, J. V., and Salmon, E. S., The fungicidal properties of certain sprayfluids. *J. Agr. Sci., 7,* 473 (1916).
15. Finholt, R. W., Weeks, M., and Hathaway, C., New theory on wood preservation. *Ind. Eng. Chem., 44,* 101 (1952).

16. Fleury, C., Action de la thio-urée sur l'*Aspergillus niger*. *Bull. soc. vaudoise sci. nat.*, *63*, 463 (1936).
17. Foster, A. A., Acceleration and retardation of germination of some vegetable seeds resulting from treatment with copper fungicides. *Phytopathology*, *37*, 390 (1947).
18. Frey-Wyssling, A., *Submicroscopic Morphology of Protoplasm and Its Derivatives*, Elsevier, Amsterdam, 1948.
19. Fries, N., and Panders, A., The growth-inhibiting effect of 5-amino-7-hydroxytriazolopyrimidine in fungi and its reversal by purines. *Arkiv. Botan.*, *2nd Ser.*, *1*, 437 (1950).
20. Gale, E. F., and Mitchell, P. D., The assimilation of amino acids by bacteria, 4. The action of triphenylmethane dyes on glutamic acid assimilation. *J. Gen. Microbiol.*, *1*, 299 (1947).
21. Gattani, M. L., Adaptation of fungi to fungicides and its significance in Agriculture. *Indian Phytopathol.*, *4*, 174 (1951).
22. Gattani, M. L., Lysis in rust uredospore germ tubes. *Phytopathology*, *42*, 70 (1952).
23. Goldschmidt, M. C., and Koffler, H., Effect of surface-active agents on penicillin yields. *Ind. Eng. Chem.*, *42*, 1819 (1950).
24. Goodwin, W., Martin, H., and Salmon, E. S., The fungicidal properties of certain spray fluids, VI. *J. Agr. Sci.*, *20*, 18 (1930).
25. Goodwin, W., Salmon, E. S., and Ware, W. M., The action of certain chemical substances on the zoospores of *Pseudoperonospora humuli* (Miy. et Takah.). *J. Agr. Sci.*, *19*, 185 (1929).
26. Görnitz, K., and Harnack, W., Zur Frage der fungiziden Wirkung von Benetzungsmitteln. *Nachrbl. deut. Pflanzenschutzdienst*, *5*, 206 (1951).
27. Greathouse, G. A., Block, S. S., Kovach, E. G., Barnes, D. E., Byron, C. W., Long, G. G., Gerber, D., and McClenny, J., Research on chemical compounds for the inhibition of growth of fungi. *Second Annual Report, 1953–1954, Contract DA-44-009-eng.-1258, Project No. 8-91-02-001, Eng. Research and Devel. Lab., Virginia* (1954).
28. Hirt, R. R., An isolate of *Poria xantha* on media containing copper. *Phytopathology*, *39*, 31 (1949).
29. Hochstein, P. E., and Cox, C. E., Effect of tetrachloro-*p*-benzoquinone on certain fermentative enzyme systems in fungi. *Phytopathology*, *42*, 11 (1952).
30. Horsfall, J. G., *Fungicides and Their Action*, Chronica Botanica Company, Waltham, Mass., 1945.
31. Horsfall, J. G., *Principles of Fungicidal Action*, Chronica Botanica Company, Waltham, Mass., 1956.
32. Horsfall, J. G., and Rich, S., Fungitoxicity of heterocyclic nitrogen compounds. *Contribs. Boyce Thompson Inst.*, *16*, 313 (1951).
33. Horsfall, J. G., and Rich, S., Fungus killers. *Yale Sci. Mag.*, *27*, 12 (1953).
34. Horsfall, J. G., and Rich, S., Effect of chemicals on sporulation of fungi. *Science*, *120*, 781 (1954).
35. Horsfall, J. G., and Rich, S., The effect of synthetic compounds on sporulation of *Monilinia fructicola*. *Trans. N.Y. Acad. Sci., Ser. II*, *18* (1955).

36. Hotchkiss, R. D., The nature of the bactericidal action of surface-active agents. (In symposium on surface-active agents.) *Ann. N.Y. Acad. Sci., 46,* 479 (1946).

37. Jurkowska, H., Investigations on the adaptability of *Aspergillus niger* to copper. *Bull. Intern. acad. polon. sci., Classe sci. math. nat. Sër. B., 4,* 4, 167 (1951). Through Abs. in *Rev. Appl. Mycol., 33,* 105 (1954).

38. Kerk, G. J. M. van der, van Os, H. C., De Vries, G., and Sijpsteijn, A. K., A new group of organic fungicides. *5th Symposium Phytopharmacie Ghent, Belgium, May 5, 1953,* 1 (1953).

39. Kittleson, A. R., A new class of organic fungicides. *Science, 115,* 84 (1952).

40. Kunkel, L. O., Physical and chemical factors influencing the toxicity of inorganic salts to Monilia sitophila (Mont.). *Sacc. Bull. Torrey Botan. Club, 41,* 265 (1914).

41. Loew, O., *Ein natürliches System der Giftwerken,* Wolf und Lüneburg, Munchen, 1893.

42. MacLeod, R. A., and Snell, E. E., The relation of ion antagonism to the inorganic nutrition of lactic acid bacteria. *J. Bacteriol., 59,* 783 (1950).

43. Macy, R., Partition coefficients of 50 compounds between olive oil and water at 20° C. *J. Ind. Hyg. Toxicol., 30,* 140 (1948).

44. Marsh, R. W., Investigations on the fungicidal action of sulphur, III. Studies on the toxicity of sulphuretted hydrogen and on the interaction of sulphur with fungi. *J. Pomol. Hort. Sci., 7,* 237 (1929).

45. Martin, H., and Salmon, E. S., Vegetable oils as fungicides. *Nature, 126,* 58 (1930).

46. Martin, H., and Salmon, E. S., The fungicidal properties of certain spray fluids, XI. Synthetic solvents. *J. Agr. Sci., 24,* 469 (1934).

47. McCallan, S. E. A., and Wilcoxon, F., The action of fungous spores on bordeaux mixture. *Contribs. Boyce Thompson Inst., 8,* 151 (1936).

48. McKee, R. K., Mutations appearing in *Fusarium caeruleum* cultures treated with tetrachlornitrobenzene. *Nature, 167,* 611 (1951).

49. Mellor, D. P., and Maley, L., Order of stability of metal complexes. *Nature, 161,* 436 (1948).

50. Müller, E., and Biedermann, W., Der Einfluss von $Cu^{+2}$—Ionen auf den Keimungsablauf von Alternaria tenuis. *Phytopathol. Z., 19,* 343 (1952).

51. Östergren, G., Colchicine mitosis, chromosome contraction, narcosis and protein chain folding. *Hereditas, 30,* 429 (1944).

52. Owens, R. G., Studies on the nature of fungicidal action, I. Inhibition of sulfhydryl-, amino-, iron-, and copper-dependent enzymes *in vitro* by fungicides and related compounds. *Contribs. Boyce Thompson Inst., 17,* 221 (1953a).

53. Owens, R. G., Studies on the nature of fungicidal action, II. Chemical constitution of benzenoid and quinonoid compounds in relation to fungitoxicity and inhibition of amino- and sulfhydryl-dependent enzymes. *Contribs Boyce Thompson Inst., 17,* 273 (1953b).

54. Owens, R. G., Metabolism of fungus spores, II. Cytochrome oxidase, succinoxidase and pyruvate carboxylose systems in homogenates of conidia of *Neurospora sitophila. Contribs. Boyce Thompson Inst., 18,* 145 (1955).

55. Raalte, M. H. van, A test for the translocation of fungicides through plant tissues. *Proc. 3rd Intern. Congr. Crop Protection, Paris, 1952.*
56. Rader, W. E., Monroe, C. M., and Whetstone, R. R., Tetrahydropyrimidine derivatives as potential foliage fungicides. *Science, 115,* 124 (1952).
57. Ramsey, G. B., Smith, M. A., and Heiberg, B. C., Fungistatic action of diphenyl on citrus fruit pathogens. *Botan. Gaz., 106,* 74 (1944).
58. Rey-Pailhade, J. de, Nouvelles recherches physiologiques sur la substance organique hydrogenant le soufre à froid. *Compt. Rend. Acad. Sci. Paris, 107,* 43 (1888). (Cited by McCallan and Wilcoxon, 1931).
59. Rich, S., and Horsfall, J. G., Metal reagents as antisporulants. *Phytopathology, 38,* 22 (1948).
60. Rich, S., and Horsfall, J. G., The relation between fungitoxicity, permeation, and lipid solubility. *Phytopathology, 42,* 457 (1952).
61. Rich, S., and Horsfall, J. G., Fungitoxicity of ethylenethiourea derivatives. *Science, 120,* 122 (1954).
62. Robbins, W. J., The pyridine analogue of thiamin and the growth of fungi. *Proc. Nat. Acad. Sci. U.S., 27,* 419 (1941).
63. Sciarini, L. J., and Nord, F. F., On the mechanisms of enzyme action, Part 22. Elementary sulfur as hydrogen acceptor in the dehydrogenations by living *Fusaria. Arch. Biochem., 3,* 261 (1943).
64. Scott, I. T., Some protein analogies of the mycelium of *Fusarium lycopersici. Missouri Agr. Expt. Sta. Research Bull., 92,* 3 (1926).
65. Shirk, H. G., and Byrne, H. E., Effect of some nitrophenols on the respiration of *Myrothecium verrucaria* spores. *J. Biol. Chem., 191,* 783 (1951).
66. Sisler, H. D., and Cox, C. E., Effects of tetramethylthiuram disulfide on metabolism of *Fusarium roseum. Am. J. Botany, 41,* 338 (1954).
67. Starkey, R. L., and Waksman, S. A., Fungi tolerant to extreme acidity and high concentrations of copper sulfate. *J. Bacteriol., 45,* 509 (1943).
68. Sussman, A. S., The effect of heterocyclic and other compounds upon the germination of ascospores of *Neurospora tetrasperma, J. Gen. Microbiol., 8,* 211 (1953).
69. Vincent, J. M., Distortion of fungal hyphae in the presence of certain inhibitors. *Nature, 159,* 850 (1947).
70. Wellman, R. H., and McCallan, S. E. A., Glyoxalidine derivatives as foliage fungicides, I., Laboratory studies. *Contribs. Boyce Thompson Inst., 14,* 151 (1946).
71. West, W., and Wolf, F. T., The mechanism of action of the fungicide 2-heptadecyl-2-imidazoline. *J. Gen. Microbiol., 12,* 396 (1955).
72. Woods, M. W., Reversible inhibition of tobacco-mosaic virus in living cells with 0.0002 molar sodium cyanide. *Science, 91,* 295 (1940).
73. Yarwood, C. E., Sulphur and rosin as downy mildew fungicides. *Phytopathology, 27,* 931 (1937).
74. Yarwood, C. E., Water content of fungus spores. *Am. J. Botany, 37,* 636 (1950).
75. Yarwood, C. E., Fungicides for powdery mildews. *Proc. 2nd Intern. Congr. Crop Protection, 1951.*

76. Yoder, D. M., Reversibility of copper toxicity to conidia of *Sclerotinia fructicola*. *Phytopathology, 41,* 39 (1951).
77. Zentmyer, G. A., Mechanism of action of 8-hydroxyquinoline. *Phytopathology, 33,* 1121 (1943).
78. Zentmyer, G. A., Inhibition of metal catalysis as a fungistatic mechanism. *Science, 100,* 294 (1944).
79. Zentmyer, G. A., and Rich, S., Reversal of fungitoxicity of 8-quinolinol and copper-8-quinolinolate by other chelators. *Phytopathology, 46,* 33 (1956).

# RECENT ADVANCES IN CONTROL OF SOIL FUNGI

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## I. Introduction

Control of plant diseases is the ultimate goal of the science of plant pathology. Methods of plant disease control are responsible largely for development of various specialized avenues of research within this science; studies have progressed far beyond the descriptive phase of diseases and inciting organisms pursued by our pioneering colleagues.

Most of the early attempts at controlling diseases were empirical, often borrowing ideas from other sciences and applying them to plant pathogens without any notion concerning their likelihood of success. Through their successes and failures many facts have been established which today have application and serve to answer some of the perplexing problems of control. The empirical approach to control problems was justified because then as well as today plant pathologists were under pressure to achieve control of diseases causing serious crop losses. There was not enough manpower or time to develop basic concepts of host-parasite-environment relationships, which occupy such important phases of any plant disease investigation of the present day, and allow the application of control measures in a much more logical manner.

The development of the field of control of soil-borne pathogens has followed precisely this procedure. The successful use of carbon disulfide as a soil fumigant for the control of grape Phylloxera in France during the latter part of the 19th century led to its trial and use for the control of soil fungi. Attempts to control grape Phylloxera by flooding were followed by attempts to control soil fungi. Both controls are still used today in limited and specific cases. Newhall (145) cites work by a number of investigators showing toxicity of chloropicrin to many microorganisms as one reason for its ultimate use in soils for control of soil fungi. Newhall again notes that methyl bromide was first used successfully as a space fumigant, and the observations of Richardson and Johnson (152) concerning its ability to penetrate soil to kill insects and nematodes led to studies concerning its use as a soil fumigant. Soil steaming was first utilized in the very late 19th century for the control of nematodes and soil insects, as described by Kühn (110) and Rudd (157). The beneficial aspects of soil steaming were soon noticed in connection with the control of damping-off diseases.

Following the successful adaptation of these and other methods to control of soil-borne pathogens, interest developed in explanations for their success as well as failures. The examination of many methods of controlling soil fungi in the light of some well defined principles of their ecology and control will be the subject of this chapter.

An early important contribution to the understanding of soil fungi was made by Waksman (194) who distinguished between a group of fungi regularly found in a given soil, termed *soil inhabitants,* and a group of fungi not a part of the regular flora, termed *soil invaders.* Reinking and Manns (151), working with the *Fusaria* in soils of Central America, applied these terms to distinguish between parasitic *Fusaria* only associated with a host and those which were present regardless of a specific host. Garrett (63) expanded their concept to include and distinguish between two contrasting types of residence by soil-borne pathogens. He defined the types as follows: *soil inhabitants,* unspecialized fungus parasites with a wide host range, whose habit of parasitism seems to be incidental to their saprophytic existence as members of the general soil microflora; *soil invaders,* more highly specialized fungus parasites whose presence in soil is dependent upon a host and who in its absence do not persist indefinitely.

An understanding of a particular pathogen with regard to its relationship with other microorganisms in a soil, and in the light of Garrett's interpretation of the distinction between the two groups enables the investigator to apply control measures in a logical rather than an empirical manner. For example, successful control of organisms belonging to the group of soil inhabitants is not likely to be achieved by exclusion methods such as the introduction of disease-free planting stock. Protective control measures must also be considered. Crop rotation with a nonsusceptible host may not result in successful control with this group of fungi either. One must think of control methods in terms of eradication of or protection against the soil inhabitants. *Rhizoctonia solani* and species of *Pythium* inciting root rot, seed and seedling decay, and *Streptomyces scabies,* the potato scab organism, are examples of pathogens belonging to this group.

On the other hand, soil invaders should be easier to control because of their dependence upon a specific host or a limited group of plants. Because of their inabililty to adapt themselves to survival and growth under a wide range of environmental conditions, often a simple alteration in cultural practice or the planting of a nonsusceptible host will be sufficient to achieve practical control. *Ophiobolus graminis*, the organism inciting the take-all disease of cereals is frequently cited as an example of this type of soil resident.

Some organisms may appear to be soil inhabitants because of their ability to remain in soils a long time, yet they do not carry on an active saprophytic existence. These organisms remain in soils for long periods as a result of their production of long-lived spores. Examples of this group are the Fusarium and Verticillium wilt-inducing organisms, the crucifer-clubroot organism, *Plasmodiophora brassicae,* and the cotton root rot organism, *Phymatotrichum omnivorum.* In a strict sense these organisms are not soil inhabitants although their persistent nature makes them resemble members of this class. In control methods, however, they respond to treatment as soil inhabitants, and are often more difficult to affect by altering the soil environment or crop sequence than organisms truly belonging to the soil-inhabitant group.

Control measures according to Walker (201) fall into one of two general categories, *prophylaxis* or *immunization.* Prophylactic measures include exclusion or eradication of the pathogen, or protection of the host against invasion by the pathogen. Immunization refers to development of disease resistance. It will not be the purpose of this chapter to present a complete history and evolution of methods used in controlling pathogenic soil fungi as this has already been done for specific phases of control by Newhall (145), Martin (123), Walker (201), and in *Soil Sci., 61,* No. 1 (1946). Instead, recent significant developments within the different phases of soil-borne pathogen control will be discussed along with interpretations of successes and failures in relation to concepts of principles involved.

The control measures will be discussed under five general topics, the first of which is *Chemical Control,* including the eradicant action of fumigants, the protective action of low- or nonvolatile materials, the chemotherapeutic action in treating diseases already established and a category of nutritional, pH, and salinity effects upon disease expression; second, *Physical Control,* including temperature, moisture, and aeration effects; third, *Biological Control*, including the effects of antibiotics, antibiosis, amendments, residues, and crop rotation; fourth, *Control* by *Disease Resistance;* and finally, *Regulatory Control.*

## II. Chemical Control

### A. ERADICANTS

Fumigation as a tool in plant disease control has been thoroughly covered in recent reviews by Newhall (144,145). In this chapter particular attention will be paid to recent research on the effect of fumigation on various components of the soil microflora in addition to the commonly noted direct effect on a given pathogen.

There are several ways in which fumigants may function in providing control of a soil-borne disease:

1. As soil sterilants, eliminating all types of microflora.
2. By selectively elminating the pathogen.
3. By reducing the inoculum potential of the pathogen and stimulating, or providing conditions that stimulate antagonistic microorganisms in the soil.

In the first approach, as with steam sterilization, the danger of severe disease development, should the pathogen accidentally be reintroduced in the absence of normal microbial antagonists, has been pointed out by several workers. The development of nutritional stresses is also common under such circumstances. Some apparent contradictions to this generally accepted theory of increased virulence of pathogens in sterilized soil have been presented by Sanford (159) and Ludwig and Henry (120). Although not substantiated by other work, Sanford reported that *Rhizoctonia solani* was less virulent in steam sterilized soil than in natural soil, a fact that he apparently associated with its vigorous vegetative growth and massing of mycelium in the sterilized soil. He reported that this pathogen was most virulent when the hyphae were young, thin, and sparse in occurrence on host tissue. Ludwig and Henry also noted that *Ophiobolus graminis* was less virulent in steamed than in untreated soil; in this case the difference was the result of the different microflora developing in the steamed soil as compared with the nonsteamed soil.

Ferguson has conducted some interesting research on controlled reinfestation of steamed or fumigated soil (59,60) by introducing nonpathogenic organisms. He added species of *Chaetomium, Pestalozzia, Myrothecium, Penicillium, Trichoderma,* and an unidentified *Botrytidae* to flats of steamed soil, and obtained retardation of spread of Rhizoctonia damping-off of peppers. This approach has many possibilities and would seem worth further extensive exploration, particularly with respect to the nursery industry.

Selective elimination of the pathogen would constitute the ideal means of successful disease control by fumigation, but practically this is extremely difficult to attain. Several workers have reported considerable variation in response of various fungi to soil fumigants; these differences are primarily quantitative, however, rather than qualitative. Chloropicrin, as Stark (171) has pointed out, is much more toxic to *Phytophthora* and *Pythium* than it is to *Verticillium* or *Armillaria,* but it is toxic to the latter two fungi if sufficiently high dosages are used. Stark noted a wide range of effectiveness of chloropicrin against 47 fungi, ranging from a concentration of 1 gram of chloropicrin in 486,000 ml. of water to kill *Pythium* sp., *Phytophthora* sp., and *Rhizopus nigricans,* to 1 to 54,000 to kill *V. albo-atrum,* 1 to 18,000 to kill *A. mellea,* and 1 to 2000 to kill *Aspergillus niger* and *Chaetomium globosum.* In this experiment the fungi were submerged in solutions of the concentrations noted for 24 hours.

Wilhelm and Ferguson (220) noted similar differential responses of fungi to fumigants when materials were applied to soil in 32-gallon cans. Concentrations of chloropicrin that killed *V. albo-atrum* did not kill *Chaetomium* spp.; *Trichoderma* spp. survived dosages of allyl bromide that were lethal to *V. albo-atrum.* The authors (233) reported significant differences in effectiveness of several fumigants against *Fusarium solani* f. *phaseoli, Phytophthora cinnamomi, Rhizoctonia solani,* and *Thielaviopsis basicola.* In general, *P. cinnamomi* was the least resistant to the fumigants, followed by *T. basicola, R. solani,* and *F. solani.*

Tests in soil in the glasshouse by Zentmyer and Klotz (234) also indicated that *Phytophthora* spp. were more sensitive to the 1,3-dichloropropene, 1,2-dichloropropane (D-D) mixture than were other components of the soil microflora.

McKeen (129) reported that in general methyl bromide was more effective against fungi than against bacteria and actinomycetes. Several fungi were appreciably resistant to the material, however, including the watermelon wilt causal organism, *Fusarium oxysporum* f. *niveum*, and *Fusarium* sp., causal organism of an onion rot root. Munnecke and Ferguson (141) demonstrated fungicidal action of methyl bromide (4 lb. per 100 cu. ft. space) in soil against *Botrytis cinerea*, *Fusarium oxysporum* f. *gladioli*, *F. oxysporum* f. *callistephi*, *Phytophthora cinnamomi*, *P. citrophthora*, *Pythium ultimum*, *Sclerotinia homococarpa*, and *Sclerotium delphinii*. In the same tests *Rhizoctonia solani* was slightly less affected and microsclerotia of *Verticillium albo-atrum* were quite resistant.

In considering the third way in which fumigants may function, recent research on effectiveness of carbon disulfide for control of Armillaria root rot is of interest. In spite of the fact, or perhaps because of the fact, that $CS_2$ is not an extremely potent fungicide it has been successful in controlling *Armillaria mellea* in citrus and deciduous orchards. Extensive research by Bliss (23) indicated that this control was not the result of direct toxicity of $CS_2$ to the pathogen, but was the result primarily of destruction of *A. mellea* through the antibiotic action of the common soil inhabitant, *Trichoderma viride*.

Bliss showed that *T. viride* developed in most cultures in which *Armillaria* had become nonviable following fumigation with $CS_2$, and was definitely antagonistic to *A. mellea* in agar culture. When roots infected with *A. mellea* were fumigated with standard dosages (302 gal/acre) of $CS_2$ in nonsterile soil containing *T. viride*, *A. mellea* was recovered after 7 days, but only *T. viride* was recovered after 32 days. In sterile soil infested with *T. viride*, *Armillaria* could not be recovered after either 7 days or 32 days even in the absence of fumigation.

A recent report by Darley and Wilbur (47) indicated that $CS_2$ is directly toxic to *A. mellea* at a dosage of 302 gal./acre. This effect was apparent on sampling roots in treated soil at intervals after treatment. *Armillaria* was recovered the first 2 to 3 days after fumigation, no organisms were recovered for the next 4 to 8 days, bacteria only were found from 30 to 50 days, then *Trichoderma* was isolated regularly. The possibility exists that, even though $CS_2$ has some direct fungicidal effect on *Armillaria*, biological control with *Trichoderma* still plays a role in further reducing the *A. mellea* population.

In addition to the correlations which undoubtedly exist between fumigation and biological control, combination effects involving nematodes and soil fungi are becoming increasingly recognized. Control of several root disease complexes with nematocides, at dosages much below the fungitoxic levels of some of these materials, has demonstrated that nematodes are providing avenues of infection for the pathogenic fungi involved. Henry (82) recently reported excellent control of an obscure root rot of southern pine seedlings with 32 gallons per acre of a 20% solution of ethylene dibromide; this indicates either that the rot was primarily nematode-induced or that nematode injury provided the only avenue of entry for possible fungus pathogens. Foster *et al.* (61) obtained better control of a black root rot of southern pine with methyl bromide than with ethylene dibromide (EDB) or 1,3-dichloropropene, 1,2-dichloropropane (D-D) indicating a nematode-fungus complex.

### B. PROTECTANTS

Soil-borne pathogens can be controlled by chemicals used in a variety of ways. First, it is often possible to eradicate the pathogen by applying volatile materials which kill the organism, as in the above discussion. These materials are for the most part also phytotoxic, so that successful application depends on the ability of chemicals to act quickly upon the pathogen and then dissipate. This leaves a soil partially or wholly sterilized and subject to the dangers of reinfestation by pathogens introduced by means of contaminated seed, planting stock, implements, or soil.

Secondly, it is possible to protect the host by chemical barriers to prevent infection of host tissue and thus exclude soil-borne pathogens. These chemicals need not act as fungicides but can provide an effective control by acting as fungistats, inhibiting fungus growth. This principle of disease control has long been applied to seeds which are subject to invasion and destruction by soil-borne pathogens. In order to act successfully in this regard it is necessary that the chemical possess little or no phytotoxicity and its effective fungitoxicity must not dissipate too rapidly.

The use of chemicals applied to soils for the purpose of protecting roots and underground stems from invasion by pathogenic soil fungi, in contrast to eradicating the pathogens has recently captured the interest of plant pathologists and chemical companies alike. The potential market for effective materials of this type is large in spite of the fact that control of seedling diseases and root rots in field plantings has remained through the years an unattained goal. Chemical seed treatments are for the most part effective only in preventing seed decay. When the young plant grows away from the protective area of the seed coat, the newly developed stem and root tissue is vulnerable to attack by soil-borne pathogens. The effective volatile fumigants used in some field, nursery, and seed-bed operations, such as chloropicrin, methyl bromide, carbon disulfide, and formaldehyde, are not adaptable to general use in the large field plantings for one reason or another.

There are a number of considerations involved in the development of an effective protectant fungicide for use in soils. These are: (*1*) general or specific effectiveness, (*2*) level of fungitoxicity, (*3*) volatility, (*4*) solubility, (*5*) effectiveness in a soil medium, (*6*) persistence or residual period, (*7*) phytotoxicity, (*8*) hazards to humans, (*9*) residue tolerances in harvested products, and (*10*) cost of manufacture.

### *1. General or Specific Effectiveness*

The potential use of a chemical for treating soil will depend upon its effectiveness in preventing growth of important pathogenic soil organisms. If a material possesses a limited spectrum of fungitoxicity it may not be successfully employed in controlling diseases incited by a complex of pathogens. For example, Kendrick and Middleton (105) showed that pentachloronitrobenzene (PCNB) inhibited but did not destroy mycelial growth of *Rhizoctonia solani* at 10 p.p.m. in laboratory tests, but under the same conditions 1000 p.p.m. had no effect upon the growth of *Pythium* and *Fusarium* species. PCNB acts as a fungistat at these concentrations. In soils containing these 3 organisms it is necessary to control all of them to prevent seed and seedling decay of beans, peas, and many other crops. Brinkerhoff *et al.* (28) report that PCNB controlled *Rhizoctonia solani* but failed to reduce seed decay and pre-emergence injury of cotton in cold wet soils. Although the pathogens were not determined it would seem likely that the seed decay might have been incited by *Pythium* species. Chemicals specifically active against a few pathogens may be potentially valuable in controlling the diseases incited by them but a limited market could make the chemicals uneconomical to produce. Mixing fungicides with overlapping fungitoxicities is a possibility for improving their individual performance. Materials with general fungitoxic capacities would offer greater promise because of a more widespread adaptation and a potentially larger market.

### *2. Level of Fungitoxicity*

The level of fungitoxicity is an important consideration because materials should be effective at relatively low concentrations to permit economical amounts of materials to be used. Considering that the top 6 inches of an acre of soil weighs approximately 2 million pounds, it would require 200 lb. of a chemical thoroughly mixed in this volume of soil to achieve a concentration of 100 p.p.m. For practical considerations of cost and handling procedures the level of toxicity should occur between 10 and 100 p.p.m. concentrations. In the light of this, it is easy to see why some materials which are very effective seed protectors are of little value when used in soil applications at these dosages. Kendrick and Middleton (105) have shown that *N*-trichloromethylthio tetrahydrophthalimide (captan) was generally active as a fungistat against a variety of soil-borne pathogens only between 100 and 1000 p.p.m. When used as a seed treatment, however, on a weight basis the concentration used is above 1000 p.p.m., where it acts very effectively as a seed protectant. When one considers the small volume of soil in relation to the seed, this concentration effect becomes even greater. Any material which requires upward of a ton per acre 6 inches to be effective is unlikely to be widely adopted for use as a soil fungicide.

### *3. Volatility*

The relative volatility of a chemical acting in a soil medium is an important consideration because it could determine the manner in which it would be applied, the general area of effectiveness, and the length of time it would remain effective. Materials which are highly volatile could be expected to affect the soil fungus population at distances farther away from the point of application than those of less volatility, providing the solubilities and soil adsorptive and absorptive capacities were equal. Such materials would be more desirable where it is necessary to treat large soil volumes effectively, as would be the case with root rots and the vascular wilt diseases of deep-rooted crops. However, it might be necessary to use an airtight seal on the soil to have the material act effectively at the upper surface. The necessity of using seals makes it unlikely that such materials can be adopted in large scale field plantings. If materials dissipate rapidly, which is desirable because of their phytotoxicity, there is danger of recontamination of the treated soil by pathogens, resulting in more serious disease losses than when they were acting in competition with the normal soil flora.

### *4. Solubility*

Most of the considerations discussed in connection with volatility can be applied to solubility of a potential soil fungitoxicant in water. Materials which are highly water soluble can usually be moved greater distances in the soil solution than those which are not. This makes them potentially of greater value for treatment of root diseases of the deep-rooted plants. On the other hand, materials of high water solubility are more easily leached away from zones where the protection is desired.

### *5. Effectiveness in a Soil Medium*

This is an important consideration for anyone who is attempting to correlate the general fungitoxicant properties of materials in tests *in vitro* with those performed in a soil environment. Kendrick and Middleton (105) and Zentmyer (230) have shown that many chemicals which are highly fungicidal on the basis of standard laboratory testing methods are ineffective when applied to soils at the same dosages. Rushdi and Jeffers (158) in studying some of the soil factors which affect the efficiency of fungicides found that soil texture, pH, and organic matter content, which in turn affect the degree of absorption, are important variables affecting the activity of fungitoxicants in soil. The action of soil microorganisms upon fungitoxicants might also be an important factor affecting activity. In this regard, Booer (25) attributes the formation of mercuric sulfide from compounds of mercury added to soil as due to the action of bacteria which give rise to the formation of hydrogen sulfide.

### *6. Persistenee or Residual Period*

This consideration is important to the chemical and physical properties of the fungitoxicant and its phytotoxic capacities. If a material has phytotoxic propensities it is essential that the persistence time in the soil be short, and its value as a protectant is correspondingly reduced. However, in order to achieve the maximum benefit from chemical treatment of soil it is desirable to have the active principle acting over a long period of time in relation to the life of the plant being protected. The persistence of action would certainly be related to solubility, volatility, and stability of the potential fungitoxicant in the soil environment.

### *7. Phytotoxicity*

This factor is one of the most important, next to the level of fungitoxicity, that determines whether or not a material will be acceptable for use in soils to control soil-borne pathogens. In the treatment of soils used for seed-beds, nursery, and glasshouse plant production, and tree sites this is an important consideration, but the soil usually can be treated long enough in advance of the introduction of plant material to allow the phytotoxic factor to dissipate. It is possible to treat field soils in advance of seeding operations with materials which are phytotoxic, as is done with nematocides. However, this requires extra expense of the application, and necessitates the use of materials which are fungicidal and possess a short residual period. The danger of recontamination of these treated soils would then be a factor to consider. There is great need of materials of low phytotoxicity and high fungitoxicity for use in controlling root diseases in established plantings of woody and herbaceous plants and in seed-furrow applications for control of post-emergence damping-off.

### *8. Hazards to Humans*

Although this is an important factor to consider, it will probably not be a limiting factor to the use of a material which satisfactorily fills other desirable criteria. Suitable precautions can be developed to handle most any hazardous chemical safely, but if these become too involved there will be a natural reluctance to use them resulting in rejection of the material or, in some unfortunate cases, human poisoning.

### *9. Residue Tolerances in Harvested Products*

The increased use of chemicals employed as pesticides on agricultural products has increased the awareness of the general public to the adulteration of food. This has resulted in the U. S. in legislation which prescribes the legal tolerance for pesticides permitted in agricultural food products. A tolerance is established as a result of studies conducted to determine levels of concentrations of pesitcides which do not produce acute and/or chronic toxicities to mammals. It will be necessary for any material showing promise as a soil fungicide to perform satisfactorily within the legal tolerances prescribed in order to be acceptable for use in agriculture.

### *10. Cost of Material*

This is a consideration which can very definitely determine how widely a material will be used. Crops producing a high income per unit area of production are able to support higher costs of pesticide use and application. In any case the cost of the materials and application will have to be more than balanced by an increased return on the harvested product due to treatment.

### *11. General Considerations*

It seems improbable that any one chemical or group of chemicals will fulfill all of the requirements for a good protectant-type chemical for use in soils. The many different types of soil-borne diseases, and the relationship of the distribution of the pathogens in the soil to host tissues subject to infection, are such that chemicals of equal fungitoxicity but different physical and chemical characteristics would not be equally effective. For example, with seedling diseases, it is necessary to confine the fungitoxicant in the upper areas of the soil where it might be easily incorporated by cultivating into the soil. Solubility or volatility would be of less importance than with materials which must protect the deep-rooted plants from infection. The latter materials are not easily incorporated deeply into the soil by mechanical means and must depend upon their diffusion rates in water and air.

When one is dealing with the problem of treating large acreages with a fungitoxicant, the techniques of application become very important. If a material is relatively inexpensive it is possible to apply it as one would spread fertilizer and mix it in by cultivation. This is done in using calcium cyanamid to control pink rot of celery incited by *Sclerotinia sclerotiorum* as reported by Middleton *et al.* (133). If a material is water soluble it might be applied in conjunction with an irrigation practice as reported by Bivins and Kofranek (21) to have been done with sodium *N*-methyl dithiocarbamate (Vapam) used to control cottony rot of column stocks, incited by the same organism.

One of the newer approaches to be employed in applying protectant chemicals to soils has been the seed-furrow technique. The technique is described by Hills and Leach (85) and Cooper (41). This method is promising for controlling post-emergence seedling diseases because effective and expensive chemicals can be placed in limited zones, in contrast to treating the entire soil volume, thus effecting a considerable saving. The object of such a treatment is to provide a zone of soil containing the protectant chemical through which the seedling emerges. Equipment to apply sprays of the chemical is inexpensive, and can be attached to the seeder so that treatment and seeding can occur simultaneously. The principle of banding treatments is not new, for it was used early in connection with the formaldehyde-drip treatments for the control of onion smut by Walker (197), but represents a new approach in the application of protectant fungitoxicants.

While the research interest is intense and the number of materials tested is large, relatively few chemicals have been adapted to commercial use as protectant-fungitoxicants, a testimony to the fact that there is much to be done in the development of this type of material. Those which have shown promise are discussed below.

### *12. Pentachloronitrobenzene* (*PCNB*)

This material was developed by I. G. Farbenindustrie and introduced in the late 1930's. However, it received little attention in the U. S. until after World War II. Last (112) reported in 1952 that it reduced the spread of *Rhizoctonia solani* in soil and since that time it has been shown by a number of investigators to be outstanding in controlling diseases incited by this organism; Moore and Conover (138), Arndt (8), Sowell (168), Campbell (33). Campbell (32) and Gallegly and Bishop (62) report that PCNB effectively controls the crucifer-clubroot organism, *Plasmodiophora brassicae.* Hooker (88) and Houghland and Cash (95) report control of the potato scab organism, *Streptomyces scabies.* This material appears to be specific in its effectiveness as reported by Kendrick and Middleton (105), showing little or no effect on *Fusarium, Pythium, Phytophthora* species, and *Verticillium albo-atrum.* Diseases incited by *Sclerotinia* spp. are reported to be controlled effectively by this chemical.

### *13. Sodium N-Methyl Dithiocarbamate* (*Vapam*)

This water-soluble compound is thought to be the decomposition product of 3,5-dimethyltetrahydro-1,3,5-2H-thiadiazine-2-thione, which was shown by Kendrick and Middleton (105) to be the most generally effective fungicide of a number tested. Zentmyer (230) reports that the 2 most effective chemicals in a laboratory assay of the effectiveness of chemicals in soil in killing *Phytophthora cinnamomi* were these 2 chemicals, which were active at 5 p.p.m. Bivins and Kofranek (21) report that in addition to controlling cottony rot of column stocks, sodium *N*-methyl dithiocarbamate controlled a number of weeds. The general fungicidal nature at low concentrations, plus its herbicidal qualities, makes it a promising new fungicide. Additional studies regarding its nematocidal qualities also show promise in this respect. The limiting feature in the use of this material as a fungicide is its phytotoxicity and the difficulty of application to large acreages.

### *14. Tetramethylthiuram Disulfide* (*Thiram*)

Thiram was developed about 1931 by E. I. du Pont de Nemours and Co., Inc. Its most widespread use has been as a seed protectant. However, it is sometimes used effectively to control the turf diseases, brown patch, incited by *Rhizoctonia solani*, and snow molds, incited by *Typhula itoana* and *T. idahoensis*, according to Howard *et al.* (97). This material has the advantage over some other more effective fungicides in possessing little or no phytotoxic properties.

### *15. Disodium Ethylenebisdithiocarbamate* (*Nabam*)

Nabam came into limited use after 1943. It is water soluble and therefore is capable of being washed into soils with comparative ease. It is often phytotoxic to plants, especially when applied to foliage, but is much less injurious than sodium *N*-methyl dithiocarbamate. The material breaks down rather rapidly in soils and therefore has a short residual period of effectiveness. Its use has been largely superseded by the zinc salt in soil as well as foliage applications.

### *16. Zinc Ethylenebisdithiocarbamate* (*Zineb*)

Zineb is only slightly soluble in water and for this reason possesses a better residual period of activity. Although considerably less effective as a fungicide than Vapam or nabam, as shown by Zentmyer in laboratory tests (230), its low order of phytotoxicity has been responsible for limited use as a seed-furrow treatment, particularly against sore-shin of cotton, incited by *Rhizoctonia solani.*

### *17. Cadmium-Containing Compounds*

This group of fungicides used principally to control certain turf diseases is represented by: Cadminate, cadmium succinate; Puraturf 177, anilinocadmium lactate; and Crag 531, copper cadmium zinc calcium chromate. According to Howard *et al.* (97) these materials are potent fungicides and cause no appreciable injury when applied to turf to control dollar spot, incited by *Sclerotinia homoeocarpa;* red thread, incited by *Corticium fuciforme;* and copper spot, incited by *Gloeocercospora sorghi.*

### *18. Mercury-Containing Compounds*

This group of fungicides has its widest use in soil treatment in control of turf diseases. These chemicals have also been employed to a large extent in treatment of some cereal and vegetable seeds as well as flower bulbs. The inorganic mercury compounds most commonly used are mercurous and mercuric chlorides. The organic mercury compounds in most common use are phenylmercury derivatives. One of the newer organic mercury compounds to show promise as a seed treatment and for controlling damping-off diseases in nursery soils when applied as a soil drench is Panogen, cyano (methylmercuri) guanidine. The mercury chlorides are still preferred fungicides for the control of brown patch and snow mold diseases of turf, while the phenyl mercury compounds are used for the dollar spot, red thread, and copper spot diseases of turf, according to Howard *et al.* (97).

For more specific recommendations concerning the fungicides used in controlling the many soil-borne diseases the reader is referred to publications by McNew *et al.* (131), Martin (124), and The American Phytopathological Society (4,5).

### *19. Seed Treatments*

The treatment of seeds by fungicides represents a very specialized and well developed approach in controlling soil-borne pathogens. Walker (201) has covered the history of the development of seed treatments from their beginning in the middle of the 18th century to the present time. There are 3 general reasons for treating seed with fungicides; first, to *disinfect* seeds that contain spores or mycelia of a pathogen inside the seed coat; second, to *disinfest* seeds which contain spores or mycelia on their surface; and third, to *protect* the seed when it is placed in the soil from invasion by pathogens contained therein.

Methods used to disinfect seeds include the use of hot water and volatile materials such as formaldehyde or ethyl mercury phosphate. Since hot water and formaldehyde treatments do not leave any residual protection on the seed, it is usually necessary to provide protection by adding a material which acts in this manner.

Chemicals used to disinfest seed for the most part also protect seed from invasion by soil-borne pathogens. Materials most commonly used both as disinfestants and protectants are: the organic mercurials, such as ethyl mercury phosphate and phenyl mercury derivatives; chloranil, tetrachloro-*p*-benzoquinone; dichlone, 2,3-dichloro-1,4-naphthoquinone; thiram, tetramethylthiuram disulfide; and captan, *N*-(trichloromethylthio)-4-cyclohexene-1,2-dicarboximide.

Chemical seed protectants and disinfestants can be applied to the seed in dust form. However, the usual practice is to use water or some sticking agents, such as a cellulose acetate sticker, to make the treatment more effective by keeping more fungicide on the seed. The slurry-method of seed treatment has been widely adopted and consists of mixing a thick water suspension of material with the seed. In recent years the practice of including insecticides, such as aldrin, dieldrin, or lindane with fungicides as seed treatments for the dual protection against soil insects and soil-borne pathogens has gained widespread acceptance. Incorporation of fungicides in seed pellets has also been used recently to a limited extent.

The reader who is interested in specific recommendations for seed treatments should consult Leukel's (116) excellent summary of this subject. In addition the references mentioned immediately above will provide more detailed accounts of materials, their dosages, and diseases controlled.

## C. CHEMOTHERAPEUTANTS

The classical approach to plant disease control has involved either eradication of the pathogen or prevention of disease (protection of the plant) by applying miscellaneous materials to the exterior surface of the plant. Little attention has been paid, until the past 10 to 15 years, to the therapy of plant disease or to the more specific field of chemotherapy, which involves action of the chemical within the host without regard to the time of infection in relation to application. Chemotherapy is a most useful term when it is applied to the use of chemicals internally for control of disease whether the materials be used as protectants, therapeutants, or eradicants.

The recent tendency to substitute the term "systemic fungicides" for "chemotherapy" seems without logical basis. Of necessity there is a distinction between translocation or systemic properties of a chemical and its action as a chemotherapeutant. Not all systemic chemicals are chemotherapeutants and conversely, not all chemotherapeutants are systemic. Some chemotherapeutants have extremely local action, which, as will be pointed out below, is not necessarily a disadvantage.

The revival of interest in this field dates back to the early 1940's. At this time Howard (96) presented some interesting concepts on chemotherapeutic control of the bleeding canker disease of maple caused by the soil fungus, *Phytophthora cactorum.* This was soon followed by research on chemotherapy for control of the Dutch elm disease and other vascular diseases by Zentmyer and Horsfall (227,92, 236,232). Developments in this field to 1948 are well summarized by Stoddard and Dimond (175). More recent, shorter review articles include information to 1952, Horsfall *et al.;* Horsfall and Dimond, Crowdy (94,90,91,44).

A number of investigations have concerned chemotherapy for control of diseases caused by soil fungi. In addition to Howard's work in 1940 on *Phytophthora cactorum,* Zentmyer and Horsfall in 1943 (232) presented evidence of the reduction of disease severity in Verticillium wilt of maple and eggplant with several organic chemicals. Dimond *et al.* (52) have published an excellent discussion of the research at the Connecticut Agricultural Experiment Station on chemotherapy for control of Fusarium wilt of tomato. Most research in this field has dealt with the vascular pathogens, *Fusarium* and *Verticillium* spp.

From the recent work in Connecticut it is evident: (*1*) that plant growth regulators in some cases cause a striking reduction in Fusarium wilt of tomato (49) and Verticillium wilt of potato (193); and (*2*) that correlation is often extremely poor between fungitoxicity of an organic chemical and chemotherapeutic activity (48,51). In the latter regard, Davis and Dimond demonstrated with Fusarium wilt of tomato that 8-hydroxyquinoline benzoate is an excellent fungicide *in vitro* but a poor chemotherapeutant *in vivo;* 4-chloro-3,5-dimethylphenoxyethanol (CC 1182) is a good fungicide and also a good chemotherapeutant; and sodium 2-benzothiazolyl thioglycolate (HD 160) is a poor fungicide but a good chemotherapeutant. Dimond and Davis (51) reported 2-carboxymethylmercapto benzothiazole to be a poor fungicide but a good chemotherapeutant for Fusarium wilt of tomato, Alternaria blight of tomato, and Dutch elm disease.

Excellent control of Fusarium wilt of tomato by the antifungal antibiotic thiolutin was reported in 1952 by Gopalkrishnan and Jump (68). Bonny Best tomato plants were placed in nutrient solutions containing graded concentrations of thiolutin (10, 20, 40, and 80 p.p.m.), then inoculated by dipping in *Fusarium* spores and transferred to pots of sand. No wilt symptoms appeared in any plants treated with thiolutin, although the fungus could be recovered occasionally from plants in the 10 and 20 p.p.m. series. *In vitro* studies showed that even 100 p.p.m. thiolutin did not inhibit growth of *Fusarium oxysporum* f. *lycopersici.* This, then, is apparently another case of increased fungitoxicity of a chemotherapeutant *in vivo.*

The significance of the chemotherapeutic activity of 2,4-dichlorophenoxyacetic acid (2,4-D) and other plant growth regulators may lie in the fact that they cause appreciable changes in host metabolism, including a decrease in reducing sugars (49). The vascular wilt diseases have been shown to be sensitive in response to changes in nutrient level of the host (202).

In general it can be said, for the vascular diseases at least, that post-infection treatments have had less effect than pre-infection treatments upon disease development. As has been pointed out by several workers, plugging of the vascular system by gums and tyloses soon after infection greatly reduces possibilities of translocation of chemotherapeutants and consequently renders post-infection treatments less effective. In injecting elm trees for control of Dutch elm disease the effectiveness of the treatment decreased directly with time following inoculation (236). In 1951 Stoddard and Dimond (176) reported the following materials as effective in preventing Fusarium wilt of carnation: 2-norcamphane methanol, 4-chloro-3,5-dimethyl phenoxyethanol, and 8-quinolinol sulfate. These materials have since been used to some extent in commercial greenhouse operations in Connecticut. Stoddard (174) also presented indications of control of red stele of strawberry when plants were treated with nabam (disodium ethylenebisdithiocarbamate) before transplanting into soil infested with *Phytophthora fragariae.*

An interesting concept in the field of chemotherapeutants in relation to root infection was presented by Keyworth and Dimond (106) who investigated the effect of root injury on development of Fusarium wilt of tomato. They demonstrated that root injury per se, whether caused by hot water treatment, root pruning, or application of various chemicals, resulted in a significant reduction in disease. This does not imply that this is the universal and only effect of successful chemotherapeutants; it indicates that caution should be exercised in assaying the effectiveness of chemotherapeutants applied to the soil or sand in the Fusarium wilt test.

The fact that the mobility of a chemical may in some cases be a disadvantage was pointed out by Chapman (35). In this work it was shown that 8-hydroxyquinoline benzoate is not adsorbed by cellulosic substances and moves through the plant to the top, when applied to the soil; it is more effective against the twig-invading *Ceratostomella ulmi* than against the root-invading *Fusarium oxysporum* f. *lycopersici.* Chapman stated that *n*-octadecyltrimethylammonium pentachlorophenate (HD 3) is tied up by cellulosic substance and does not move out of the roots when applied as a soil treatment; this material is more effective against *F. oxysporum* f. *lycopersici* than against *C. ulmi.* The concept seems to have merit; it is unfortunate that Chapman used as an example a material (HD 3) that Keyworth and Dimond later showed to be effective against the tomato wilt organism because of the root injury factor.

Zentmyer in 1954 (229) attempted to control a root-rotting fungus (*Phytophthora cinnamomi*) by application of chemotherapeutants to the foliage or stems of plants. Striking, but temporary, reduction of wilting of avocado seedlings was obtained when plants were carefully sprayed with solutions of 8-hydroxyquinoline benzoate so that material did not enter the nutrient solution in which the plants were growing; no direct chemical evidence of translocation was demonstrated. Recent reports of downward translocation of several antibiotics (73) and carbamates (136) indicate that there may be possibilities in this approach to root disease problems.

Indications of the possibilities in this approach are also seen in the report by Halleck and Cochrane (77) of the effect of fungistatic agents applied to the leaves of bean plants on the bacterial flora of the rhizosphere. They found that applying Bordeaux mixture, malachite green, and zineb (zinc ethylenebisdithiocarbamate) to the foliage of young bean plants resulted in significant reduction in numbers of bacteria in the rhizosphere. No evidence was obtained as to what part of the molecule moved through the plant to the roots.

## D. NUTRITION

The use of fertilizers to control pathogenic soil fungi is for the most part an indirect and not an altogether successful approach, inasmuch as the influence is largely upon the host. The net effect is usually the production of a host plant, which by virtue of being in a state of accelerated and vigorous growth, is more capable of productivity in the presence of disease-inciting organisms than one suffering from nutritional deficiencies. The effect of vigorous growth resulting from adequate fertilization sometimes enables the host to pass quickly from a susceptible into a resistant stage as far as infection is concerned. It is reported (1) that high soil fertility shortened the period of susceptibility to infection by some soil-borne pathogens of sugar beet seedlings. The host thus escapes infection because the environment is less favorable for the pathogen than it is for the host. Nutritional influences, therefore, may result in disease control by protecting the host from severe disease expression or by allowing it to escape infection.

The truth in the foregoing statements has provided a basis for some nonprofessional people to become adherents to the idea that if plants are kept in a state of perfect nutritional balance there will be no diseases. Such a dogma has no sound foundation in fact; indeed, there are numerous examples of obligate parasitism where the most vigorous and seemingly healthy appearing plants are the only ones suffering from diseases.

Since McNew (130) has recently summarized the present status of knowledge concerning the effect of fertility upon disease development, it will not be necessary to cover this subject extensively here. A few examples will suffice to illustrate the principles involved.

Nitrogen, potassium, and phosphorus are the three elements which most often affect development of plant diseases. One clear-cut illustration where fertility level markedly affected the severity of a soil-born disease is reported from Montana involving damping-off of sugar beet seedlings infected by one or more of the following fungi:

*Phoma beta*, *Pythium* spp., and *Rhizoctonia* spp. (1). The application of phosphorus and nitrogen fertilizers to soils of low fertility resulted in more vigorous seedling development and approximately 50% reduction in seedling mortality. Such spectacular results are rare. Hooker (87) reported that root rotting of corn incited primarily by *Pythium* species was more severe in soils of low fertility and that the effect of fertility was largely upon the host rather than the parasite. One must be aware of all the factors operating before attributing successful control of soil-borne diseases to a fertility program. A plant disease is the result of an interaction between two biological systems, one parasitizing the other and both are susceptible to the influences of the environment. The environment in turn is composed of individual components subject to influence by each other, so the entire relationship becomes interdependent and complex.

The Wisconsin school of plant pathologists, following L. R. Jones' initial investigations of this complex relationship, have studied the problem with controlled experiments particularly with reference to the vascular diseases of vegetables. Walker (200, 201) summarizes some of this work and concludes that while it is possible to show marked effects of host nutrition upon disease development in nutrient culture work, there is very little basis to hope that host nutrition per se will solve many of the disease control problems, because of the many modifying factors. One of the best known examples of disease control by fertilizer application is the case of Fusarium wilt of cotton, incited by *Fusarium oxysporum* f. *vasinfectum.* Neal (142) showed that application of potassium salts reduced the severity of the disease in sand cultures and in the field. Inconsistent results in the field by other investigators led to further exploration and resulted in Miles (134) and Young and Tharp (224) reporting that this reduction occurred only in soils low in potassium content and with plants moderately resistant to the disease. When very susceptible varieties were examined, no appreciable reduction of disease occurred in potassium-deficient soils with additions of potassium fertilizers.

Similarly, Leach and Davey (114) reported a reduction in the severity of Sclerotium rot of sugar beet, incited by *Sclerotium rolfsii*, by the application of nitrogenous fertilizers in soils where the infestation was moderate. Heavily infested fields showed no such benefit from this practice, however.

A complex relationship between fertility level, particularly nitrogen, and the host and pathogen is illustrated by the take-all disease of grain. Garrett and Mann (65) reported that the locking up of the available nitrogen supply in the decomposition process of grain stubble during the fall and winter did not reduce the level of infestation of the organism by starvation sufficiently to prevent the reoccurrence of the disease in spring-sown grain when it too was grown at a low level of nitrogen. However, a winter cover crop of trefoil, which competed with the organism for the available nitrogen and caused a reduction in its level of infestation, contributed readily available nitrogen in the spring when it was plowed under prior to seeding with grain. The available nitrogen was of more benefit to the rapidly growing crop than to the take-all organism, which had been reduced by nitrogen-starvation.

McNew (130) has made some cautious generalizations regarding the relationship of the fertility and disease development. He states that damping-off and root rots are often reduced in severity by promoting disease-escaping growth habits, such as rapid development of roots. Nitrogen and phosphorus are the two most important elements in this regard and, therefore, it is essential that these two elements be in proper balance to be beneficial.

The wilt diseases are most critically affected by the ratio of nitrogen and potassium, he states. These effects are for the most part only modifying effects and cannot radically alter the response of a particular host to a disease, because plants that are inherently immune or very susceptible to a given disease will react accordingly in spite of the nutrient balance. The plants of intermediate susceptbility will show responses more clearly.

Because of the complex relationship of nutrient level and disease development, each disease situation must be considered alone with no assurance that the findings will have general applications under all conditions where the disease might occur. For this reason there has been little work of major importance developed recently showing nutrient application as a satisfactory means of soil fungus control.

## E. SALINITY

The total concentration of salts in the soil solution has been shown to exert an influence upon disease development, particularly upon the seedling decays. The effect is for the most part upon the host in rendering it more or less susceptible to infection so that the disease-escaping mechanism is at work in this regard. Beach and Chen (17) showed that soluble salt concentrations sufficiently high to injure the rootlets of seedlings did not appreciably alter the occurrence of Pythium and Rhizoctonia damping-off diseases in tomatoes, and that these organisms readily attacked the injured seedlings. It has been noted by the authors in some of the saline soils of California that Rhizoctonia root canker of lima bean develops much more severely in areas in the field where the plants are stunted by high salinity.

Beneficial effects of increased solute concentration were reported by Smith and Walker (165) in connection with Aphanomyces root rot of pea, incited by *Aphanomyces euteiches*. They achieved complete control of the disease in the greenhouse by increasing the concentration of the balanced nutrient supply. However, Walker and Hare (203) found that this was only a temporary control when applied to the field. Walker *et al.* (205) reported that a nutrient culture study of Verticillium wilt of tomato, incited by *Verticillium albo-atrum*, showed that the severity of the disease was increased by raising the concentration of the balanced nutrient solution. Christensen *et al.* (37), on the other hand, report just the opposite results in connection with Verticillium wilt of cotton, incited by the same organism.

Anderson (7) reported that the number of *Phytophthora cinnamomi* infected pineapple roots decreased in a solution culture study as the total nutrient salt concentration in solution was increased up to about 1400 p.p.m. He concluded, however, that this would not be a practical means of control of the disease because the soils of Hawaii were subject to the leaching action of high rainfall, and it would not be possible to maintain the high salt concentration in the root zone.

As a means of practical control of a root disease, except in the case where it is possible to leach soils of high salt concentration to achieve more vigorous host growth, manipulation of salinity does not seem to be a practical means of controlling soil-borne diseases.

### F. SOIL REACTION

Soil reaction plays a part in influencing the severity of a number of soil-borne diseases, but there are only a few striking cases where control of a disease is achieved by altering soil pH.

One of the earliest and most extensively studied soil-borne diseases to be adequately controlled by adjusting soil pH is the potato scab disease, incited by *Streptomyces scabies.* Halsted (78) reported in 1895 that the application of sulfur to soils successfully controlled this disease. The practice of applying sulfur to reduce soil pH is still used in controlling this disease and the related sweet potato scab disease (149,89). Inhibition of growth of the organism under conditions of high acidity was originally thought to be the main effect of control by sulfur applications. Although there is variation among different isolates of this organism with regard to their tolerance to pH levels, the majority of them are limited in growth below pH 5.0. Since Gillespie and Hurst (67) examined many different soils and came to the conclusion that potato scab rarely occurred in soils with a pH of 5.2 or lower, the commonly accepted recommendation is for sulfur application for scab control. The high buffering capacity of some soils, particularly in some areas of California, makes the application of sulfur impractical for control of this disease in those areas. Several tons per acre are often required to lower the pH sufficiently to achieve control.

Daines (46) notes conflicting reports from various investigators with regard to control of potato scab by sulfur applications. It appears that the variation in control occurs more frequently in neutral or highly buffered soils than in those naturally slightly acid. Evidence supporting the belief that the pH effect is not solely a direct one upon the organism is presented in the work of Schroeder and Albrecht (164) and Gries *et al.* (76). They showed that scab infection increased as either calcium or potassium was increased in the presence of an adequate supply of the other. Scab was least severe when the two elements were in balance. Horsfall *et al.* (93) recently presented data to show that potato scab-incidence is related to the calcium content of the tuber which is governed by the content of replaceable calcium in the soil, which is in turn governed by hydrogen ion concentration and calcium dosage. This theory may account for the apparent discrepancies occurring in sulfur applications and failure to control scab. Gries (75) suggests that part of the beneficial effects of sulfuring soil to lower the pH and subsequently control the disease is due to the increased solubility of aluminum ions which are toxic to the organism.

It has been suggested that lack of control by sulfur applications in some instances can be attributed to the presence of strains of the organism which tolerate a lower pH. Smoot (167) cites evidence for this claim by reporting on two strains of the sweet potato scab organism, *Streptomyces ipomoea.* He found one which grew well at pH values of 4.5 to 5.0, in contrast to the common strain which failed to grow below pH 5.3. Waksman (195) had reported a similar situation existing in strains of *Streptomyces scabies.*

The crucifer-clubroot organism is ordinarily not active in soils with a pH of 7.0 or higher. The liming of soils for the control of this organism has been a common practice although at times it has failed. Wellman (213) attributed the control to the toxic action of the hydroxyl ion upon the organism. Gries *et al.* (76) state that the calcium-potassium balance was important in the control of the organism, because at any level of pH where the concentration of the calcium ion increases the disease decreases. It is quite likely that both effects may play parts as pointed out by Walker (201), depending upon the conditions of the soil with particular reference to aeration and moisture.

Garrett (64) has listed a number of diseases favored by either acid or alkaline soils. Recently Bingham and Zentmyer (20) presented evidence to show that Phytophthora root rot of avocado, incited by *P. cinnamomi,* was decidedly less severe in nutrient cultures with a pH of 4.0 or lower. The host was found to grow acceptably well at pH rates as low as 3.5. They later showed (231) that the toxicity of nitrite to the organism increased at pH 4.5 compared to pH 6.5, while the host was not appreciably damaged by nitrite at the lower pH. It is interesting to speculate that field control of this disease by lowered pH might be related to the formation and greater toxicity of the nitrite ion to the causal organism, although this was not the case in nutrient culture studies.

Kincaid and Gammon (107) reported recently that the incidence of tobacco blackshank, incited by *Phytophthora parasitica* var. *nicotianae,* increased as the pH of the soil progressed from acidity toward neutrality.

It seems quite likely that further development in the use of pH adjustment in soils for the control of soil-borne diseases will involve a clearer understanding of the basic effects upon nutrient availability and the metabolism of the host and parasite. The relatively wide range of tolerance to pH change possessed by most pathogens residing in the soil medium makes this approach toward control rather unattractive to investigators interested in soil fungus control.

## III. Physical Control

### A. TEMPERATURE

There are several ways to use temperature to control soil-borne pathogens. The most common and effective way is to raise the temperature to or above the thermal death point of the organisms. In a soil medium this is accomplished by steam heat (free-flowing or under pressure), by electrical heat, by burning combustible material on the soil surface, or by solar radiation. When the pathogen is present in or on a seed, tuber, bulb, or rhizome, it is sometimes possible to raise the temperature of the propagative organ sufficiently with hot water or dry heat to kill the pathogen without injuring the host. Eradication of the pathogen is thus accomplished.

A less direct method of using temperature to control soil-borne diseases involves the mechanism of escape. Susceptible crops planted at temperatures which are unfavorable for growth of the pathogen, but not for the host plant, will very often escape serious damage. In actual practice this can be accomplished either by adjusting the planting dates to take advantage of warm or cool soils, which favor the host but not the pathogen, or by planting in climatological areas unfavorable for the pathogen but favorable to the host.

The subject of soil-steaming and -heating for the control of soil fungi has been discussed adequately by Johnson (100), Newhall (145), and Baker and Roistacher (12) and all have included extensive bibliographies upon this subject. It is unnecessary to discuss this phase of temperature control in any detail. Although heating soil by various means to control soil fungi is for the most part an efficient means of control, there are certain limitations and dangers. Cost of generating steam to treat soil adequately, frequently limits this practice to use in glasshouses and plant beds of moderate size. Since this treatment usually kills most soil microorganisms, the danger of reinfestation is great. This effect is recognized by noting that diseases sometimes develop to a greater extent in pasteurized than in nonpasteurized soils due to the rapid growth of some pathogens in the absence of competing and/or antagonizing organisms. If soil pasteurization is used as a means of controlling soil-borne pathogens it is important to maintain a pasteurized medium by using sterilized containers and pathogen-free planting stock. A practice which has not received much attention but would seem to offer considerable merit in protecting pasteurized soil from reinfestation by pathogenic organisms is the introduction into treated soil of microflora known to be antagonistic to specific disease-producing organisms (59,60).

Pasteurized soils, particularly those subjected to prolonged periods of soil steaming, may contain a variety of detrimental influences both physical and chemical. These are, as elucidated by Johnson (100), the partial decomposition of organic and inorganic materials resulting in the formation of substances toxic to plants, particularly ammonia; modification of the nitrogen cycle in soils; and a modification of the physical structure and capacities of the soil. The net result upon certain plants susceptible to these changes such as tomatoes, tobacco, soybeans, and cowpeas, is a retardation in seed germination and plant growth. These adverse effects can be avoided by using a soil mixture for nursery culture which is free of readily decomposable organic matter as described by Matkin and Chandler (125).

Beneficial results noted, above those resulting from the elimination of a pathogenic organism, arise from liberated nutrients from organic and mineral constituents and subsequent modifications resulting from increased microbial activity according to Johnson.

Numerous devices using steam or electricity for pasteurizing soil have been developed and evaluated. These are thoroughly discussed by Newhall (145) and Baker and Roistacher (12) for the reader who is interested in this subject.

The use of heat to kill fungi associated with seeds was successfully used by Jensen in Denmark in 1888 according to Walker (201). He immersed wheat and barley seed in water at 132°F. for 15 minutes and eliminated the loose smut organism, *Ustilago tritici*. The practice has been adopted for control of certain other fungi which infect seed, such as *Rhizoctonia solani* in pepper seed, Baker (10), and the celery late blight organisms, *Septoria apii* and *S. apii-graveolentis*, Bant and Storey (14). Bever (19) reports that a 4-hour soaking period for wheat seed prior to heating the seed in hot water is not always long enough to allow killing of the mycelium of the loose smut organism because of the differential rates of water absorption between varieties. A 6-hour soaking period, followed by 10 minutes at 130°F. was found to eliminate all signs of infection.

Success in controlling *Fusarium oxysporum* f. *gladioli* infections of gladiolus cormels is reported by Bald and Markley (13), Roistacher (154), and Roistacher *et al.* (155).

Cooley and Kushman (39) reported on the use of dry heat for controlling black rot of sweet potatoes incited by *Endoconidiophora fimbriata* by curing the potatoes at 110° and 105°F. for 24 hours or at 110°F. for 16 hours.

This control mechanism on seeds and planting stock is one of disinfection or eradication of the pathogen. The necessity for using this means of treatment is dictated by the fact that spores or mycelium of some fungi are associated with internal plant tissues. The elimination of the pathogen by this means, however, does not protect the seed or planting stock from recontamination, just as the pasteurization of soil does not prevent reinfestation. Therefore, in order to effect control of the soil fungi, it is often necessary to provide additional protection to these parts by means of surface protectants or to introduce the disinfected material into pasteurized soil.

Taking advantage of soil temperature influences upon growth of hosts and their pathogens sometimes offers the easiest and most efficient way to control a disease. Many of these relationships are discussed by Walker (201). In general, a group of diseases incited by the Fusarium wilt-inducing organisms are favored by warm soil temperatures. These diseases with certain exceptions are found to a greater extent in the middle and southern regions of the U. S. than in the northern regions, where cooler soil temperatures prevail. For example cabbage yellows, incited by *Fusarium oxysporum* f. *conglutinans* and Fusarium wilt of tomato, incited by *F. oxysporum* f. *lycopersici* cause no serious damage out-of-doors in the northern regions of the U. S.

In these cases the causal organisms produce their maximum vegetative growth between 25° and 28°C. and these are the soil temperatures where maximum disease occurs. When the optimum temperature for growth differs between host and pathogen, effective control is often achieved. This is true of the cabbage yellows disease where cabbage grows well at 20°C. and the pathogen produces maximum growth at 28°C. Cabbage grown between 15° and 20°C. does not show severe manifestations of the disease because the host grows better than the pathogen at these temperatures. The reverse is true with Fusarium wilt of cotton, where the host grows well at temperatures above the optimum for the growth of the pathogen, *F. oxysporum* f. *vasinfectum*, and plants often escape infections at high soil temperatures.

It is difficult to separate the influence of soil temperature upon host and pathogen. Since both are biological entities reactive to temperature, it is necessary to consider the influence upon both. A clearer case of the temperature influence upon host susceptibility is exhibited by seedling blight of wheat and corn, incited by the same organism, *Giberella zeae.* Dickson (50) determined that seedling blight of corn developed best from 8° to 20°C. and not at all above 24°C., while seedling blight of wheat developed best at temperatures from 12° to 28°C. Little or no blighting of wheat developed below 12°C. Wheat growth is favored by relatively low soil temperatures, while corn develops best at relatively high soil temperatures. The pathogen grows over a wide range from 3° to 32°C. Disease development in these two instances was poorest at temperatures which were most favorable for host growth. Eckerson and Dickson (54) later showed that this temperature influence upon host susceptibility was due to influence upon the chemical composition of cell walls and the nature of food available to the pathogen.

Leach (113) has made an interesting study of soil-temperature influences upon host and pathogen in relation to prediction of the occurrence of pre-emergence damping-off diseases. Using *Rhizoctonia solani* and *Pythium ultimum* as pathogens and watermelon and spinach as hosts he calculated the ratio between the coefficient of velocity of emergence of the seedlings and growth rates of the pathogens at different temperatures. When the ratio fell below 1, in other words, the growth of the pathogen was greater than that of the host, severe pre-emergence damping-off occurred. With values between 1 and 4 more post-emergence disease developed. He states that for the diseases studied this ratio was inversely related to the severity of pre-emergence infection. This concept explains why high-temperature crops like cotton, cowpeas, lima beans, and peanuts are more subject to seed decay or pre-emergence damping-off at low temperatures, while low-temperature crops like spinach and peas are more subject to these diseases at high soil temperatures, providing soil moisture conditions were similar. As pointed out by Leach, a factor complicating this relationship would be the existence of pathogen strains differing in optimum temperatures for growth but possessing similar pathogenicities. This has been shown to be the case by Kendrick (104) in lima bean root rot incited by *Rhizoctonia solani* in a planting date study. He found that this disease developed similarly in different locations at different soil temperatures. The range of temperatures where the disease developed was 15° to 35°C. A study of the optimum temperatures for growth of different strains of the pathogen showed a variation between 18° and 33°C. The disease followed closely the temperature optimum of the specific pathogenic strain.

Waggoner and Shaw (192) pointed out that the temperature of the host tissue and adjacent soil may be considerably more temperate than either air or soil surface temperatures would suggest, and thus the host climate may be more favorable to disease development than that of the surrounding air.

Some hosts have a restricted time of susceptibility to infection and if the environment is not favorable for infections during this period the host escapes the disease. This situation is clearly demonstrated in the onion smut disease. Infection must take place through the developing cotyledons below ground. After the cotyledons mature they are no longer susceptible to invasion by the pathogen. Optimum temperatures for the germination of the pathogen's chlamydospores, secondary spores, and mycelial growth lie between 13° and 22°C. (201). Above 25°C. the pathogen shows little activity. Onion seeds planted in soils at 25°C. or above, as they are in the southern part of the U. S., escape infection. Those planted in northern U. S. soils in the cool spring are subject to infection and must be protected by chemicals. Similar temperature relations exist with wheat bunt. Early fall sowings of wheat in the Pacific Northwest have less disease than late fall sowings, and in other sections late spring-sown wheat shows less infection than the early spring-sown wheat. By manipulating the planting date to take advantage of soil temperatures favorable for the host at the expense of the pathogen relative control is achieved.

### B. MOISTURE AND AERATION

Soil moisture is a factor of considerable and varied influence on soil fungus problems. Excess moisture can either favor disease, as in the case of the water molds, or drastically reduce disease, as exemplified by flooding for control of Fusarium wilt of banana and several other diseases. On the other hand, reduced soil moisture can also result either in increased or decreased disease development.

From the aspect of disease control, high soil moisture is effective in suppressing several different types of diseases. Flood-fallowing has been the principal method used since 1939 by the United Fruit Company in Honduras to reclaim land made unsuitable for further banana production by high incidence of infestation with *Fusarium oxysporum* f. *cubense.* Stover's interesting recent research (180,178,179,181, 182,177) has provided valuable information on the various factors involved in the control of banana wilt by flooding. In studying several species of *Fusarium,* including *F. oxysporum* f. *cubense,* in the laboratory, Stover (178) found that the *Fusarium* population decreased appreciably when soil was maintained at moisture levels over 25% saturation, was greatly reduced at moisture contents from 50 to 80% of saturation, and was almost nonexistent at complete saturation. This indicates that the *Fusarium* spp. tested were strongly aerobic, as Garrett (64) has stated to be the case for root-infecting fungi, and indeed fungi in general. Additional tests by Stover demonstrated that *F. oxysporum* f. *cubense* persisted a relatively short time (less than 14 weeks) in field soils maintained under anaerobic conditions (179).

It is evident from this research and from earlier reports of successful growth of bananas for a number of years following replanting flood-fallowed areas that this practice significantly reduces the population of the banana wilt organism in the soil. Stover has pointed out, however, two important factors involved in recolonization of these lands by *F. oxysporum* f. *cubense:* the dispersal of spores of this organism by river water used to fill or replenish the basins, and the often appreciable survival of the fungus in the oxygen-containing surface layer of submerged soil (180). It has been recently shown (180) that *F. oxysporum* f. *cubense* can survive in the surface inch of submerged soils for 6 to 12 months or more, whereas it is eliminated from lower depths in much shorter periods. This inoculum in the surface-oxidized layer of mud therefore provides a source of reinfestation for the field when it is drained and replanted to bananas; that the inoculum potential in some cases may be extremely low and reinvasion involves a considerable period of time are indicated by the frequent success of flood-fallowing. A means of eradicating or further reducing the population of *F. oxysporum* f. *cubense* while it is still confined to a vulnerable position in the surface layer is certainly indicated.

Several other examples of disease control by flooding the soil may be cited. Brooks (29) reported destruction of over 90% of the sclerotia of *Sclerotinia sclerotiorum* in Florida by flooding muck soil for 6 to 8 weeks during the summer. Moore in 1949 (137) further investigated the matter of eliminating this fungus by flooding, finding that 100% of the sclerotia of *S. sclerotiorum* decayed within 23 to 45 days in flooded soil. Addition of green organic matter to the soil surface did not accelerate sclerotial rotting; the fact that these experiments were conducted during the winter months, with relatively low temperatures, may account for this discrepancy, as otherwise, it would seem likely that stimulation of other microorganisms by such organic additions would hasten rotting.

White rot of onion, incited by *Sclerotium cepivorum*, is another example of a disease that is suppressed by high soil moisture (198). Whether this is the result of rotting of sclerotia, some other effect on the fungus, or the result of increased host resistance at the higher moisture levels is not known.

What appears to be an anomalous situation in control of one of the water molds appears in the report by Valleau *et al.* (189) of elimination of *Phytophthora parasitica* var. *nicotianae* in several cases by flooding infested tobacco fields. This is apparently further indication of the fact that *Phytophthora* spp. are not anaerobic organisms, as emphasized by data of Curtis and Zentmyer (45) on infection of avocado seedlings by *P. cinnamomi* at various oxygen levels. This fungus was found to be most effective in attacking avocado roots at the highest oxygen level (6.8–8 p.p.m. $O_2$), and ineffective at low oxygen levels.

Rhizoctonia damping-off is another common disease inhibited at high soil moisture levels, as Roth and Riker (156) demonstrated in their work with pine seedlings. In this case, Rhizoctonia damping-off reached a peak when the soil moisture level rose to 68% saturation, then the disease incidence dropped off sharply as saturation increased to 100%. On the other hand, damping-off caused by *Pythium irregulare* increased directly with increase in moisture content of the soil, and was highest at 100% saturation. Beach more recently (16), has presented similar results with Rhizoctonia damping-off of tomato seedlings, and Pythium damping-off of peas.

A different type of effect of moisture on soil-borne diseases is seen in Tyner's water-soak method (188), for control of several grain diseases. In this work it was demonstrated that loose smut of barley incited by *Ustilago nuda* could be as effectively controlled by soaking the seed in water for 56 to 64 hours as by soaking in 0.2% Spergon suspension for 40 hours, preceded by a 6- to 8-hour water-soak. Hebert

(80) reported that the length of the anaerobic treatment necessary for control was inversely related to temperature. Loose smut of wheat was also eliminated by a 48-hour water-soak, without addition of fungicide. Similar striking control of the internally borne seed pathogen of barley, *U. nuda,* has been reported by Leben and Arny (115). In research on similar control of *Ascochyta pisi* infection of pea seed, Wallen and Skolko (206) speculated that control might result from the action of antifungal antibiotics produced by the natural bacterial flora of the seed during the soaking and drying period. This control of *A. pisi* was negated when soaked and dried seed were dipped in 2% chlorine. Chlorine was shown to inactivate several antibiotics *in vitro.* Other possible explanations for the effectiveness of the water-soak method are the direct effect on the fungus and/or the production of antifungal metabolic products by the seed itself (206).

Instances of reduced soil moisture's controlling soil fungi are less common and less spectacular than the effects noted above of excess moisture. In many cases merely a temporary reduction in disease incidence or a slower development of disease is noted, as exemplified by several recent papers on *Phytophthora* spp. (83,55,187,235). Zentmyer (228) has reported elimination of *Phytophthora cinnamomi* from naturally infested sandy loam soil by drying to 1% soil moisture at temperatures below the maximum tolerated by the fungus.

In any consideration of the effect of soil moisture on disease control or disease development, aeration is obviously also an important factor. Aeration effects cannot be overlooked, as has been indicated in the discussion of banana wilt, and various Phytophthora-induced diseases. Hutchins (98) emphasized the tremendous numbers of microorganisms, as well as higher plants, competing in the soil for oxygen and pointed out the numbers of factors that may reduce oxygen content and cause plant injury. These same factors in many cases are also deleterious to pathogens in the soil. In this respect however, Zak (225) in research on little-leaf disease of pine stated that poor soil aeration did not appear to affect the pathogenicity of *Phytophthora cinnamomi* except perhaps by predisposing roots to attack. Research on this same fungus on avocado by Curtis and Zentmyer (45), and Zentmyer and Bingham (231) indicates that predisposition is unnecessary for ready infection.

## IV. Biological Control

### A. GENERAL

It is in the realm of soil-borne diseases, whether they be caused by fungi, bacteria, actinomycetes, or viruses, that the principle of biological control has the greatest application in relation to plant diseases. The teeming and exceedingly varied microbial population of the soil, affected so strikingly by factors of pH, moisture, aeration, and nutrition, provides an extraordinarily complex subject for study and analysis. In the struggle of these microorganisms for survival, which essentially implies competition for food, many associative and antagonistic effects result, as Waksman pointed out in 1941 (196) in his thorough review of microbial antagonism.

Intimately involved in these effects, as noted by Weindling in discussing association effects of fungi (209) are myriads of metabolic products of fungus activity, as well as of activity of other types of soil microorganisms. These products may have a stimulating, toxic, or indifferent effect upon fungi and other organisms.

The fact that microorganisms produce substances that are toxic or antagonistic to each other has been recognized for some 70 years. However, the concentration of tremendous research effort on these antagonistic chemicals, and the development of this approach into the field of antibiotics is one of the striking phenomena of the past 10 years. Stimulated by the success of penicillin in the medical field and somewhat more indirectly by the development of the sulfa drugs, great strides have been made in the isolation, identification, and application of these antibiotic materials in relation to biological control of plant disease.

In the field of microbiology and the study of antibiotic substances the pioneering work of Waksman and Dubos is too well known to need review in this chapter. There have been several recent comprehensive reviews of this field. Hoogerheide (86) summarized the early work in relation to bacteria. Benedict in 1953 (18) presented a comprehensive review of the antibiotics produced by actinomycetes. The general topic of control of plant diseases by use of antagonistic organisms has been well covered in recent articles by Stallings (170) and Wood and Tveit (223).

## B. ANTIBIOTICS (SPECIFIC)

There has been considerable recent work on the effect of specific antibiotic substances on growth of various soil fungi. This in large degree has followed Weindling's (207,208) original contribution on parasitism of *Rhizoctonia solani* by *Trichoderma viride* and the isolation of toxic substances involved. This work was a landmark in the history of antibiosis in relation to plant disease.

It is not the intention in this chapter to review all of these contributions, however, some of the more significant ones will be cited. In general, most of the antibiotics studied to date are relatively ineffective in control of soil fungi. However, during the past 2 or 3 years there has been increasing emphasis on development of antifungal antibiotics.

Gliotoxin, produced by *Trichoderma viride* and *Gliocladium* sp., was one of the earliest antifungal antibiotics reported (211,208). It has the empirical formula $C_{14}H_{16}N_2S_2O_4$, with a molecular weight of approximately 347. This material is toxic to the hyphae of *Rhizoctonia solani* at dilutions of 1–300,000. Control of Rhizoctonia damping-off of several plants has been reported by adding *T. viride* to the soil (207,212,56,3). It seems likely that the control of damping-off is related directly to the production of gliotoxin in the soil. Although Evans and Gottlieb (56) could not detect the antibiotic in nonsterile soil infested with *T. viride*, but could detect it in sterile soil similarly infested, damping-off was controlled to some degree in both soils. The failure to recover gliotoxin in nonsterile soil was undoubtedly the result of rapid inactivation of the material by other microorganisms, but a continuous production of extremely small amounts of gliotoxin may still account for the disease control obtained.

Actidione (cycloheximide) is an effective antifungal antibiotic: 3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide. Gottlieb *et al.* (72) reported Actidione as inhibitory to a number of fungi, including several soil organisms (*Pythium graminicola, Fusarium oxysporum* f. *lycopersici*, and *Rhizoctonia solani*) at 10 p.p.m. It has been reported by Vaughn (190) and Vaughn and Klomparens (191) as highly effective in controlling turf diseases including dollar spot caused by *Sclerotinia homoeocarpa.* The effectiveness of materials such as Actidione is reduced by the influence of the normal soil microflora, which cause rapid disappearance of many antibiotics from soil. Gottlieb (70) noted rapid inactivation of Actidione in nonsterile, but less effect in sterile soil. If it were not for this degrading of antibiotic substances, seeding soil with antagonistic organisms or periodic applications of the materials themselves would have a much better possibility of controlling various soil invaders.

Another antibiotic that has shown some promise against soil fungi is thiolutin: 6-acetamido-4-methyl-1,2-dithiolo-[4,3-B]-pyrrole-5-(4H)-one. As noted in the section on chemotherapeutants, Gopalkrishnan and Jump obtained good control of Fusarium wilt of tomato with thiolutin. It has been reported as a fairly effective fungicide against *Phytophthora cinnamomi* in soil (230).

A new antifungal agent, filipin, was reported by Gottlieb *et al.* in 1955 (71) to be produced by an unidentified actinomycete. In a subsequent report by Ammann *et al.* (6) the actinomycete, from Philippine soil, was named *Streptomyces filipinensis* n. sp. Filipin is an unsaturated substance of the conjugated polyene type, and is toxic to a wide range of fungi pathogenic to plants and animals, including such soil fungi as *Sclerotinia sclerotiorum, Rhizoctonia solani,* and *Fusarium oxysporum* f. *dianthi.*

One of the most recent examples of an effective antifungal antibiotic is the material oligomycin, developed at the University of Wisconsin (166). Oligomycin is produced in submerged culture by a species of *Streptomyces,* has the empirical formula $C_{25}H_{40-42}O_7$, and has shown high fungitoxicity *in vitro* to a number of plant pathogens, including *Charlara quercina, Verticillium albo-atrum, Colletotrichum circinans,* and *Pythium debaryanum.* Newburgh and Cheldelin (143) reported oligomycin to be highly fungicidal also to *Tilletia caries.*

Other antibiotics effective against fungi, but not subjected to extensive tests for control of soil fungi include griseofulvin, produced by *Penicillium griseofulvin;* anisomycin, produced by *Streptomyces griseolus;* and mycostatin, produced by *S. noursei.* Griseofulvin is rare among antibiotics in containing chlorine; it has the formula $C_{17}H_{17}O_6Cl$ (27).

Several reports of bacteria antagonistic to *Rhizoctonia solani* have appeared (43,53) but the toxic principle has not been identified.

## C. ANTIBIOSIS

In addition to the reports noted above of toxic effects of specific antibiotics on soil fungi, a tremendous amount of literature has accumulated on antagonism of various microorganisms to soil-borne pathogens. Most of this work has not progressed to the stage of isolating chemicals responsible for the antagonism, but several interesting relationships are revealed. Since the pioneering researches of Henry (81) and Sanford and Broadfoot (162) on the effect of various soil inhabiting microorganisms on the pathogenicity of *Ophiobolus graminis*, a wide range of antagonists and pathogens has been investigated.

The activity of a number of soil organisms against several pathogens, including *Rhizoctonia solani*, *Actinomyces scabies*, *Ophiobolus graminis*, *Phymatotrichum omnivorum*, and *Pythium* and *Phytophthora* spp. was well summarized in 1946 by Sanford (160), and in 1955 by Wood and Tveit (223). Two facts seem particularly noteworthy in this regard: (*1*) the relatively high percentage of normal soil inhabitants that have antagonistic capabilities, and (*2*) the lack of direct correlation of numbers of antagonistic organisms in a soil with disease control.

Cooper and Chilton (42) in 1950 reported that the percentage of isolates of actinomycetes with antibiotic activity against *Pythium arrhenomanes* ranged from 18.5 to 31.5% in the sugar cane soils of Louisiana; this was based on 8302 cultures. In further research in Louisiana, Luke (121,122) noted that from 15.4 to 17% of a large number of soil fungus isolates were also antagonistic to *P. arrhenomanes*. These included species of *Aspergillus*, *Penicillium*, and *Spicaria*.

Johnson (101) tested a number of actinomycetes and fungi antagonistic to *P. arrhenomanes* in agar cultures for their ability to inhibit development of Pythium root rot of corn in the greenhouse. Significant control was obtained with several of these which had displayed the highest antagonism in culture. The lack of direct correlation between antibiotic activity in agar culture and in soil was seen in the fact that many highly antagonistic isolates had no effect on root rot development. A later report by Johnson (102) indicates that the lack of activity in soil of some isolates that are excellent antagonists *in vitro* may be the result of differential inactivation of antibiotic substances by components of a clay soil.

Other workers have also noted the frequent lack of correlation between activity of antagonists in culture or in sterile soil as compared with activity in nonsterile soil (74). The necessity for considering other influencing factors is strikingly indicated in the reports of control of Rhizoctonia damping-off of citrus by *Trichoderma lignorum* (208,212). Control was obtained only in the presence of *T. lignorum* when the soil pH was sufficiently low. Aytoun (9) reported that *Trichoderma* effectively antagonized *Armillaria mellea* in culture only at an acid pH and speculated that field control of *Armillaria* by *Trichoderma* could only occur in acid soils.

Meredith (132) isolated a number of actinomycetes and several fungi that were antagonistic to *Fusarium oxysporum* f. *cubense* in one type of soil agar, but not in a different soil agar.

Morton and Stroube (139) noted that rather small percentages of bacteria, actinomycetes, and fungi isolated from Louisiana soils were antagonistic in culture to *Sclerotium rolfsii*. While not all of the antagonistic isolates were tested for disease control, several significantly reduced disease development.

Of significance in the complex interaction of antagonists and pathogens in the soil is the status of the pathogen as a soil inhabitant or soil invader. Wilhelm (219,217) notes that *Verticillium albo-atrum* is a soil invader which has difficulty in permeating nonsterile soils, and is readily killed or greatly retarded in its pathogenic activity by such fungi as *Gliocladium*, *Chaetomium*, *Stachybotrys*, and *Myrothecium*. Lachance and Perrault (111) have noted more direct evidence of antagonism against another vascular pathogen, *Fusarium oxysporum* f. *lini*. They isolated a number of bacteria and actinomycetes from Canadian soils which were antagonistic to *F. oxysporum* f. *lini* in culture; the majority of these at least retarded symptoms of wilt on flax seedlings in greenhouse soils.

Thornton (185) has pointed out that several factors may be involved in antibiosis. He reports work by F. A. Skinner showing that *Streptomyces albidoflavus* can affect *Fusarium culmorum* by direct attack on the hyphae, production of antibiotic substances, or competition for nutrients. It is evident that the standard antibiotic screening methods will not detect organisms that are effective antagonists by one of these other two mechanisms.

## D. AMENDMENTS, RESIDUES, CROP ROTATION

The topics of crop residues, crop rotation, and organic soil amendments are all closely related, and involve, to a large degree, antibiosis or the activities of antagonistic microorganisms. It is conceivable that in some cases of disease control involving organic amendments factors other than antibiosis are involved. These other factors may include nutritional deficiencies occurring in the presence of a nonhost plant or in the presence of an organic amendment which may result in a different rhizosphere microflora (160). Toxic products formed directly from the decomposition of the amendment may also play a part in disease control.

Rotation of crops is an often used and a frequently abused recommendation made in connection with the control of diseases incited by soil-borne pathogens. It is usually assumed that the continual cropping of a single host or group of susceptible host plants will provide a favorable medium for increasing the inoculum potential of a given pathogen. This is very often the case but there are numerous exceptions to this rule which demand further explanations. Likewise, it is often assumed, although sometimes erroneously, that removing the susceptible host will reduce the inoculum potential of the pathogen. A number of studies have shown why some soil-borne pathogens are reduced by rotation and others are not.

Garrett's (64) development of Waksman's concept of two groups of soil-borne pathogens; the soil "invaders" and the soil "inhabitants," as pointed out earlier, provides a fruitful approach to the study of biological control. Soil invaders, being well adapted to a parasitic existence within the plant but incapable of successful competition with other organisms in the soil, should be particularly sensitive to antagonism by various saprophytes, and thus may be relatively easy to control by biological means. Soil inhabitants are capable of saprophytic activity in the absence of host roots, and thus are much more difficult to control biologically.

Before any intelligent recommendation of the crop rotation practice for control can be made it is necessary to consider whether the particular pathogen belongs to the soil invader or soil inhabitant category. Within the group of soil invaders it is important to determine whether or not an organism possesses resistant spore or mycelial structures. The chance that the mere lapse of time between plantings of susceptible crops will be an effective means of eradicating the pathogen is good only with the group of pathogenic soil invaders without resistant spores or mycelia. These pathogens are usually characterized by being specialized parasites, with a restricted host range. Examples of this group are the cabbage blackleg organism, *Phoma lingam,* which is successfully eliminated from contaminated soil by a

3- to 4-year rotation with noncruciferous crops according to Walker (201), and *Ophiobolus graminis* the cereal take-all organism, which Garrett (64) concludes can usually be adequately controlled by a 1-year rotation with any noncereal crop except pasture. Garrett has considered *Verticillium albo-atrum* as a representative of this class on the basis of work done on potatoes by McKay (128) and on black raspberries by Zeller (226). They showed that infections from this organism dropped off markedly after a 3- and 4-year rotation with nonsusceptible crops. Evidence that this may not be the case is presented by Wilhelm (218), who showed that this fungus was capable of surviving by means of dried microsclerotia for 13 years in culture. From a field planted to grains and pasture for 8 consecutive years following 1 year in tomatoes he was able to demonstrate the presence of the pathogen in 50% of his soil samples assayed by growing tomato seedlings in the samples. When the field was assayed in the same manner 14 years after the original tomato crop, he recovered the pathogen in one of his samples, and concluded that a noticeable reduction of the inoculum potential occurred between the ninth and fourteenth year. In light of this evidence it would seem that this organism should probably belong to the class of soil invaders which are able to persist in soils for long periods of time by means of resistant structures.

Further examples of the soil invaders which survive by means of resistant spores or sclerotia are *Plasmodiophora brassicae,* the crucifer-clubroot organism; *Aphanomyces euteiches,* the pea root rot organism; and *Phymatotrichum omnivorum,* the cotton root rot organism. These organisms are difficult to eliminate from the soil merely by avoiding planting the susceptible hosts for several years. A minimum of 5 years between cruciferous crops is recommended for the reduction of the inoculum potential of *P. brassicae,* although even longer periods have sometimes failed to reduce the disease to a point of economical unimportance. Garrett (64) states that the length of rotation required to keep clubroot under control is probably correlated with the suitability of the soil for parasitic activity.

Linford and Vaughan (118) cite evidence that *A. euteiches* was able to survive and cause serious damage to peas even with a lapse of 10 years between pea plantings. Linford (117) later reported that the fungus might survive in the absence of the pea host by acting as a weak parasite on the roots of apparently resistant crops and weeds.

The survival period of *Phymatotrichum omnivorum* in the absence of susceptible hosts is directly related to the length of time that sclerotia remain viable in the soil. In contrast to the sclerotia of *V. albo-atrum*, Taubenhaus and Ezekiel (184) and others found that sclerotia of *P. omnivorum* lost their viability more rapidly in dry than in moist soil. A number of discrepancies between the results of investigators studying the survival of sclerotia of this fungus seemed to indicate that an important factor in survival was whether or not soil moisture and aeration conditions were such to promote spontaneous germination of the sclerotia. Whenever spontaneous germination occurred, considerable reduction in the number of sclerotia was noted. Under field conditions it seems to be generally believed that a minimum of 4 years between cotton crops is necessary to show a good reduction of the disease. Streets (183) indicates that a 5-year rotation with flax, early guar for seed, or guar or sesbania as green-manure crops reduced severe root rot of cotton to a trace in the succeeding crop. It is likely, however, that the green-manure crops might have had an effect upon the germination of the sclerotia over and above the fact that they served as nonhost plants for the organism.

The partial eradication, by crop rotation, of soil-borne pathogens which belong to the soil inhabitant class presents an entirely different problem. Because these organisms are facultative parasites or saprophytes, a lapse of time between plantings of susceptible hosts may or may not reduce the inoculum potential to a point where economical control of disease is achieved. The potato scab causal organism (*Streptomyces scabies*), and *Rhizoctonia solani* (incitant of root and stem cankers of many crops) are typical examples of fungi belonging to this class. Goss and Afanasiev (69) report that 2-year rotations were ineffectual in reducing the incidence of potato scab, but that 4- to 6-year rotations gave satisfactory reductions. There are known instances in California where land not previously cultivated has produced potatoes affected by scab, thus giving evidence that this organism exists as a part of the natural soil flora. It is quite likely that soil conditions particularly in relation to the pH of soils are important in influencing the persistence of this organism in the absence of susceptible hosts. Goss and Afanasiev (69) also report that a 4-year rotation was sufficient to reduce the level of *Rhizoctonia solani* in soils to a point where tuber scurf occurred at a negligible minimum. Blair (22) showed that the activity of *Rhizoctonia* was reduced by the addition of 1% or more of ground dried green manure, which apparently favored the growth of competing organisms more than the pathogen. Evidence that this is not a general rule or perhaps specific types of green manure may sometimes favor *Rhizoctonia* rather than depress it is presented by several workers. Goss and Afanasiev (69) report that damping-off of sugar beets increased following an alfalfa crop, and Boyle (26) found that the incorporation of soybean hay into sandy soil from which all other organic matter had been sifted enhanced the growth of *Sclerotium rolfsii* and *Rhizoctonia* sp. When all of the organic matter was removed, the establishment of these 2 organisms was poor. Boyle concludes from this evidence that the removal of organic matter from the upper level of soils is of greater importance in controlling these parasites than selection of crops in a rotation program. In attempts to isolate *Rhizoctonia solani* from soils known to be infested by this organism, the writers have noted that when the pathogen is obtained from soil platings it is always associated with bits of organic debris. This would seem to be additional evidence to Boyle's assumption regarding the importance of organic materials in maintaining *Rhizoctonia* in soils.

Organisms inciting Fusarium wilt diseases are examples of pathogens possessing extreme longevity in soils. For example, Barker (15) reports that rotations of from 10–12 years between flax plantings are necessary to avoid losses from wilt (incited by *Fusarium oxysporum* f. *lini*), and Jones and Gilman (103) recorded a survival period of 14 years for the cabbage yellows organisms, *Fusarium oxysporum* f. *conglutinans*.

Since all of the foregoing discussion involves the principle of eradication of the pathogen it is important to consider an additional precaution in regard to the ultimate control of these fungi. This is a precaution to prevent the introduction of pathogens into soils where effective reductions in the inoculum potentials have been accomplished by rotation practices. This is accomplished by using seed, planting stock, and organic amendments known to be pathogen-free. If this additional precaution is not taken the effectiveness of a good crop rotation practice may never appear.

The above discussion has considered only the effect of the absence of the susceptible host upon the survival of the pathogen in relation to the capacity of the organism to exist independently in a soil medium. In recent years the important influence of crop sequence and organic amendments upon the other soil microflora has come to be recognized. A complete understanding of these influences may provide a means of using crop rotations successfully in cases where previously they were of little value.

There are several ways in which a particular crop sequence may influence the inoculum potential of a pathogenic soil fungus. The preceding crop although not adversely affected by the pathogen, may provide the means for increasing its development by acting as a host or releasing nutrients favorable for pathogen growth upon decomposition. This would seem to be the case reported by Afanasiev and Morris (2) where seedling diseases of sugar beets incited by species of *Aphanomyces* and *Rhizoctonia* increased following plantings of alfalfa. Goss and Afanasiev (69) also reported this fact. Results of a crop sequence experiment in relation to the survival of *Rhizoctonia solani*, as yet unpublished by the senior author, confirm the influence of alfalfa in maintaining a high level of inoculum potential for this organism. On the other hand, in the same experiment purple vetch, barley, and oats were found to reduce the subsequent disease assayed on the roots of baby lima beans. This effect may be similar to that reported by Garrett (64) in the role of trefoil which depleted nitrogen from soil during the winter months thus hastening the starvation of *Ophiobolus graminis.* Kommedahl and Young (109) recently reported that corn stalks in the soil increase the likelihood of subsequent seedling blight of wheat, incited by species of Fusarium, but decrease the chances for infection by *Rhizoctonia solani.* Sanford (161) showed that maize meal additions to soil caused a growth suppression of *Rhizoctonia solani* through increased activity of *Trichoderma viride.* When living potato plants (a host for Rhizoctonia) were present in soils with maize meal added, Rhizoctonia persisted. This pathogen was found to die out of soils when wheat, oats, barley, or corn were cultivated, but to persist when potatoes, beans, or peas were cultivated. Sanford concluded, therefore, that *Rhizoctonia solani* depends entirely on parasitic nutrition for its persistence in soil. These results would appear to support the view of Blair (22), that increased activity of microorganisms able to utilize certain amendments caused a nitrogen starvation of the mycelium of the pathogen. Thus, it is clearly evident that the type of organic matter present in the soil is an important influence upon the subsequent development of a given pathogen.

A second way in which crop sequence or the application of organic amendments may influence the development of a soil-borne pathogen is the effect upon populations of microorganisms in the rhizosphere which may be antagonistic to a given pathogen. This subject has received intensive investigations by a group of Canadian workers (214,186) and is reviewed by Sanford (160). Although Wood and Tveit (223) have recently reviewed the subject it will be well to examine a few examples of the effect of crop sequence or organic amendments upon soil microflora in relation to control of some of the parasitic diseases incited by soil-borne pathogens. Wood and Tveit report that Rhizoctonia seedling disease of radish was not reduced in sterile soils where a variety of organic amendments were added but was when antagonists were added with the amendments. In nonsterile soils the addition of the amendments alone effected a marked reduction in the disease. West and Hildebrand (214,84) in a study of the strawberry root rot complex found that soybean grown as a cover crop and then incorporated into infested soil caused a striking reduction in the incidence of the root rot and a shift in the bacterial equilibrium of the soil. When red clover was tried in the same manner, they found no effect upon the subsequent disease or general microflora of the soil. Oswald and Lorenz (148) noted a reduction in potato scab development derived from growing soybeans in the field as a green-manure crop preceding a crop of potatoes. Chinn *et al.* (36) reported that adding soybean meal to soil resulted in germination of conidia of *Helminthosporium sativum,* which was followed by lysis of germ tubes as a result of antibiotic substances in the soil. In apparently a more direct effect on the antagonistic microflora, McGahen (127) noted that the antibiotic index of sugar-cane soil was increased by bagasse compost, cowpea and soybean trash, and was decreased by bonemeal, bloodmeal, and tankage.

Wilhelm (215,216) reported that a number of organic amendments reduced the inoculum potential of *Verticillium albo-atrum* in the soil. They included bloodmeal, fish meal, ammonium sulfate, and chopped alfalfa hay. He noted that the effect was roughly proportional to the nitrogen content of the amendments. Zentmyer reported (228) that alfalfa meal (ground stems and leaves) had a striking effect in control of Phytophthora root rot of avocado in glasshouse and some field experiments. This effect was obtained with applications ranging from 5 to 20 tons of dry alfalfa meal per acre, and has been attributed to both a quantitative and a qualitative change in soil microflora. Great variations in response of different field soils indicate the importance of the latter factor.

The evidence available certainly indicates that the influence of crop rotation or sequence upon subsequent disease development is complex and related to a number of factors. One must consider whether a pathogen is eliminated from a soil environment merely because it requires the unique nutrients of the host it parasitizes and in the host's absence dies, or whether it is more directly affected by the activities of microorganisms in the rhizosphere or soil beyond. Under the latter influence it is possible to have two effects, that of increased activity of the rhizosphere or general soil microflora competing more favorably than the pathogen for the available nutrients, or the formation by soil microorganisms of metabolites which act as toxins and poison the pathogen. With an increased understanding of the influence of the microorganisms and nutrients of the rhizosphere and soil in relation to factors which affect them and in turn influence invasion and parasitism by soil-borne pathogens, it is possible that biological control of soil-borne diseases can be advanced rapidly.

## V. Disease Resistance

The control of soil-borne pathogens by developing host resistance achieved notable early success in the field of plant pathology. In fact, Orton's success (147) at the beginning of the 20th century in developing resistance to 3 major Fusarium wilt diseases served as the foundation for other disease resistance programs. The watermelon variety, Conqueror, resulting from a cross of the African citron and watermelon, has served as a major source of resistance to *Fusarium oxysporum* f. *niveum*, the watermelon wilt organism. This was the first disease resistant material produced by hybridization, with the transfer of resistance as the primary purpose. Varieties of cowpea, cotton, flax, tomato, and cabbage resistant to Fusarium wilt diseases were soon developed.

Although many programs for developing resistance in plants have been successful, they have been so largely through empirical testing of varieties and selections. A better understanding of the nature of disease resistance in most instances would perhaps enable the plant breeder to select individuals possessing certain morphological or physiological characteristics known to resist or neutralize the manifestations of pathogenesis.

Excellent discussions of disease resistance in plants have been presented by Walker (199,201) and Wingard (221,222).

Orton (147) early recognized different types of disease resistance. He termed them "disease-escaping," "disease-enduring," "disease-resisting," and "immune varieties." The disease-escape mechanism sometimes operates as a result of the protecting nature of the environment which has been discussed in another section. For example, early maturing varieties of cowpeas and cabbage, susceptible to Fusarium wilt diseases, often escape infection because of soil temperatures unfavorable to growth of the pathogen but not the host.

The distinction between protective and exclusion mechanisms in the control of a soil-borne pathogen depends upon whether the terms are defined from the viewpoint of the pathogen or host. In other words a host may have a protective mechanism which enables it to exclude the pathogen and by virtue of this mechanism it is resistant to the disease incited by the organism. Protective mechanisms may be mechanical or physical barriers to pathogen invasion which prevent wetting and subsequent spore germination. Examples of these mechanisms acting against air-borne pathogens are listed by Wingard (221,222) and Walker (201). A few clear cut examples of mechanical protection against soil-borne pathogens are described below. The cotyledons of the common onion are susceptible to invasion by the smut organism, *Urocystis cepulae*, only in the seedling stage. The cotyledons of the Welch onion have a much shorter period of susceptibility and therefore this variety is regarded as resistant. Evans (57,58) concludes that this resistance is due in part to the characteristics of the outer cotyledonary membrane. Smut resistant corn varieties owe their resistance in part to the protective nature of leafsheaths covering nodal tissue susceptible to fungus penetration.

Mishra (135) recently cites evidence in support of Hawkins' and Harvey's (79) theory that resistance of the potato variety, White McCormick, to parasitism by *Pythium debaryanum* is due to the inability of the parasite to penetrate cellular membranes by mechanical force. He was unable to demonstrate the production of pectin enzymes by this organism in sufficient quantity to be responsible for cell wall dissolution. However, the question might be still unresolved because he gives no hint with regard to differing tensile strengths of cell membranes of varieties differing in susceptibility to attack by this organism. It is conceivable that the host-parasite reaction might still involve the production of chemicals necessary for dissolution of cell membranes in susceptible varieties.

The best known example of host protection by chemical exclusion of soil-borne pathogens occurs in the case of onion smudge as reported by Walker *et al.* (204), and Link and Walker (119). Colored varieties were shown to contain the water-soluble phenolic compounds, protacatechuic acid, and catechol, which diffuse out of the scale into moisture droplets and prevent germination of spores of the causal fungus, *Colletotrichum circinans.* White varieties of onions do not contain these compounds and are therefore susceptible to invasion as are the inner white scales of the colored varieties which do not possess these materials.

In 1927 Klotz (108) suggested that disease resistance in plants might be associated with the ability of the host to neutralize enzymatic action of pathogens. Recently Johnson and Schaal (99) and Schaal and Johnson (163) have reported that the resistance of certain varieties of potatoes to infection by the scab organism, *Streptomyces scabies,* is associated with the presence of *o*-dihydroxyphenol, chlorogenic acid, and tyrosinase in the periderm around lenticels of the tubers. They concluded that in invaded tissue chlorogenic acid is oxidized to quinones, which in *in vitro* tests were found to be toxic to the growth of *Streptomyces scabies.* They likewise report that chlorogenic acid and other *ortho*-dihydricphenols, which are substrates for tyrosinase, hasten the rate of suberization of potato tuber tissue. While the evidence strongly suggests chemical toxicity between host and pathogen, it is not conclusive and resistance may be associated with increased tuber suberization.

Walker (201) illustrates the complexities, which arise in attempts to attribute chemical resistance of host cells to particular materials or groups of compounds, by citing the example of the presence of volatile sulfur compounds in the fleshy uncolored scales of the onion, which are toxic to onion bulb pathogens *in vitro,* but fail to prevent infection. The fungus parasites apparently are capable of neutralizing the toxic effects of these materials. Further evidence against assuming that a toxic host material is associated with resistance is the example of the volatile sulfur compound, allyl isothiocyanate, present in black mustard. This compound is quite toxic to the clubroot organism, *Plasmodiophora brassicae, in vitro,* and Rochlin (153) reported that it was responsible for the resistance of this plant to the clubroot organism. Pryor (150) produced resistant black mustard plants without detectable isothiocyanates by growing them in nutrients low in sulfur. These plants retained their resistance to the clubroot organism. Stahmann *et al.* (169) analyzed naturally occurring resistant and susceptible black mustard plants for the presence of isothiocyanates and found no differences. These findings tend to discredit the original claim concerning the role of isothiocyanates in relation to resistance to the clubroot organism.

Workers, who are apt to associate resistance with toxins present in a host on the basis of *in vitro* tests, should first consider whether toxic materials exist in a toxic state within host cells, and whether or not the presence of the host is capable of neutralizing the action of the toxin.

Eradication of a soil-borne pathogen by action of the host is shown in certain potato varieties resistant to wart, incited by *Synchytrium endobioticum.* In this instance, potato tissue, by virtue of being extremely sensitive to infection is killed rapidly in advance of invasion. Since the parasite requires living tissue upon which to grow and survive it soon dies for lack of living host cells. Thus the hypersensitivity of host tissues is responsible for eradicating the parasite.

Physiological and mechanical resistant mechanisms are intimately related to the interesting study of the physiology of parasitism, for which there is an abundant source of literature. The reader who is interested in pursuing this subject as well as disease resistance further, is invited to read the following summaries and discussions: Walker (199,201), Wingard (221,222), Bloch (24), Brown (30,31), Gäumann (66), Coons (40), Stevenson and Jones (173), Clayton (38), Stevenson and Akeley (172), and Munger and Newhall (140).

## VI. Regulatory Control

### A. QUARANTINE

It is possible to limit the spread of certain soil-borne pathogens from one geographical area to another by legislative restrictions upon the movement of soil and plant parts from an area of contamination into areas free of the pathogen. This control mechanism is an example of exclusion. It is accomplished by quarantining infested areas to confine pathogens therein, and/or by confiscating and destroying contaminated materials intercepted at the frontiers of the pathogen-free areas. Although this is a well established and successful means of controlling diseases of man, animals, and plants, it has not had too much application or success in restricting the development of soil-borne diseases. It is quite likely that a number of pathogenic soil fungi could have been confined to their sites of origin if movement of contaminated plant parts from area to area had been known to be an important means of disease dissemination when new agricultural areas were being developed. It would also have been necessary to have legislation and enforcing agencies operative at the time of these agricultural expansions. Since this was not the case, particularly during the development of agriculture in the U. S. many of the important soil-borne pathogens are found in widely distributed areas. In areas where they do not occur it is not unlikely that they were introduced but failed to become established because of some unfavorable conditions of the environment.

Plant quarantine restrictions in the U. S. were applied by individual states at first. Although attempts were made as early as 1892 to secure Congressional action in this regard, it was not until 1912 that the Plant Quarantine Act was passed. California led other states in the U. S. in this regard by passing a quarantine act in 1881; Germany had an act in force in 1875, and Australia in 1877. The quarantine act of California which applies to movement of certain plant materials into and within the state may be responsible for confining the important cotton root rot organism, *Phymatotrichum omniverum*, within a few areas outside the major cotton producing areas of the state.

Potato wart, incited by *Synchytrium endobioticum*, was probably introduced into the U. S. on potatoes imported from Europe during the winter of 1911–12. The disease was subsequently found in Pennsylvania in 1918, as well as in West Virginia and Maryland. Because of the serious nature of the disease, the 3 states immediately instigated an embargo against shipment of potatoes from the affected regions. Subsequent studies have shown that the organism survives only in soils where the temperature does not exceed 70–72°F. This fact has permitted the mapping of areas where the organism is likely to persist. The limitations of the environment and the quarantine of affected areas have kept this organism under control to the present time.

The practice of returning soil accumulated at sugar beet loading stations along railroad sidings in California was found to be a means of distributing *Sclerotium rolfsii,* incitant of Sclerotium rot of sugar beet, into noninfested areas. Prompt action in preventing this practice has stopped this means of disseminating the organism.

Examples of controlling the spread of soil-borne pathogens by legislative means are not numerous and are not likely to be. Quarantine regulations against old and established diseases would be unjustified except in cases where new agricultural areas were being developed and it could be clearly shown that these areas were free from the pathogens. Before quarantines will be enacted upon a national scale the following conditions would have to be met in regard to the disease under consideration: (*1*) a disease of national importance in relation to its potential destructiveness, (*2*) information concerning its distribution, conditions for its existence and dissemination and life history available, (*3*) no other available reasonable means for its control, (*4*) state or local agencies are not better able to handle the situation, and (*5*) a natural feeling of apathy and resentment against regulation overcome. These are cited by McCubbin (126) as reasons why a number of disease situations have not been put under Federal control.

For the reader who is interested McCubbin (126) has prepared a very complete analysis of the plant quarantine subject.

The principles involved in restricting the movement of plant materials and the use of a certification program to insure clean planting stock can be a very effective means of controlling soil-borne pathogens. For nursery-grown plants this necessitates the use of uncontaminated soil, containers, and plant materials. Baker (11) and Chandler (34) have emphasized this point of pathogen exclusion, particularly in relation to the high-value ornamental nursery industry. A good summary of the clean stock program for ornamentals is contained in the report of the Committee on Seed and Plant Material Certification of the American Phytopathological Society (146).

## References

1. Afanasiev, M. M., and Morris, H. E., *Phytopathology*, *32*, 477–86 (1942).
2. Afanasiev, M. M., and Morris, H. E., *Phytopathology*, *40*, 963 (1950). (Abs.)
3. Allen, M. C., and Haenseler, C. M., *Phytopathology*, *25*, 244–52 (1935).
4. American Phytopathological Society, *Agr. Chemicals*, Jan., Feb., and Mar. 1954.
5. American Phytopathological Society, *Agr. Chemicals*, April, May, and June 1955.
6. Ammann, A., Gottlieb, D., Brock, T. D., Carter, H. E., and Whitfield, G. B., *Phytopathology*, *45*, 559–63 (1955).
7. Anderson, E. J., *Phytopathology*, *41*, 2 (1951). (Abs.)
8. Arndt, C. H., *Plant Disease Reptr.*, *37*, 397–400 (1953).
9. Aytoun, R. S. C., *Trans. Proc. Botan. Soc. Edinburgh*, *36*, 99–114 (1953).
10. Baker, K. F., *Phytopathology*, *37*, 912–24 (1947).
11. Baker, K. F., *The Florists' Rev.*, *102*, 31–2 (1948).
12. Baker, K. F., and Roistacher, C. N., *Calif. Agr. Expt. Sta. Manual*, in press, 1957.
13. Bald, J. G., and Markley, B. B., *Phytopathology*, *45*, 693 (1955). (Abs.)
14. Bant, J. H., and Storey, I. F., *Plant Pathol.*, *1*, 81–3 (1952).
15. Barker, H. D., *Minn. Agr. Expt. Sta. Tech. Bull. 20* (1923).
16. Beach, W. S., *Penn. State Coll. Bull. 509* (1949).
17. Beach, W. S., and Chen, S. Y., *Penn. Agr. Expt. Sta., Bull. 434* (1942).
18. Benedict, R. G., *Botan. Rev.*, *19*, 229–320 (1953).
19. Bever, W. M., *Phytopathology*, *41*, 875–9 (1951).
20. Bingham, F. T., and Zentmyer, G. A., *Phytopathology*, *44*, 611–14 (1954).
21. Bivins, J. L., and Kofranek, A. M., *Calif. Agr.*, *9*, No. 9, 6 (1955).
22. Blair, I. D., *Ann. Appl. Biol.*, *30*, 118–27 (1943).
23. Bliss, D. E., *Phytopathology*, *41*, 665–83 (1951).
24. Bloch, R., *Phytopathology*, *43*, 351–4 (1953).
25. Booer, J. R., *Ann. Appl. Biol.*, *31*, 340–59 (1944).
26. Boyle, L. W., *Phytopathology*, *46*, 7–8 (1956). (Abs.)
27. Brian, P. W., *Ann. Botany (London)*, *13*, 59–77 (1949).
28. Brinkerhoff, L. A., Oswalt, E. S., and Tomlinson, J. F., *Plant Disease Reptr.*, *38*, 467–75 (1954).
29. Brooks, A. N., *Phytopathology*, *30*, 703 (1940). (Abs.)
30. Brown, W., *Brit. Mycol. Soc. Trans.*, *19*, 11–33 (1934).
31. Brown, W., *Botan. Rev.*, *2*, 236–81 (1936).
32. Campbell, L., *Plant Disease Reptr.*, *38*, 283–5 (1954).
33. Campbell, L., *Plant Disease Reptr.*, *38*, 859 (1954).
34. Chandler, P. A., *Pacific Coast Nurseryman*, *12*, 15–16, 44–5 (1953).
35. Chapman, R. A., *Phytopathology*, *41*, 6–7 (1951). (Abs.)
36. Chinn, S. H. F., Ledingham, R. J., Sallans, B. J., and Simmonds, P. M., *Phytopathology*, *43*, 701 (1953).
37. Christensen, P. D., Stith, L. S., and Lyerly, P. J., *Plant Disease Reptr.*, *38*, 309–10 (1954).
38. Clayton, E. E., *Phytopathology*, *43*, 239–44 (1953).
39. Cooley, J. S., and Kushman, L. J., *Phytopathology*, *41*, 801–3 (1951).

40. Coons, G. H., *U. S. Dept. Agr. Yearbook 1953*, pp. 174–92.
41. Cooper, W. E., *Phytopathology, 44*, 331 (1954). (Abs.)
42. Cooper, W. E., and Chilton, S. J. P., *Phytopathology, 40*, 544–52 (1950).
43. Cordon, T. C., and Haenseler, C. M., *Soil Sci., 47*, 207–15 (1939).
44. Crowdy, S. H., *Empire J. Exptl. Agr., 20*, 187–94 (1952).
45. Curtis, D. S., and Zentmyer, G. A., *Am. J. Botany, 36*, 471–4 (1949).
46. Daines, R. H., *Soil Sci., 61*, 55–66 (1946).
47. Darley, E. F., and Wilbur, W. D., *Phytopathology, 44*, 485 (1954). (Abs.)
48. Davis, D., and Dimond, A. E., *Phytopathology, 42*, 563–7 (1952).
49. Davis, D., and Dimond, A. E., *Phytopathology, 43*, 137–40 (1953).
50. Dickson, J. G., *J. Agr. Research, 23*, 837–70 (1923).
51. Dimond, A. E., and Davis, D., *Phytopathology, 42*, 7 (1952). (Abs.)
52. Dimond, A. E., Davis, D., Chapman, R. A., and Stoddard, E. M., *Conn. Agr. Expt. Sta. Bull. 557* (1952).
53. Dunleavy, J., *Phytopathology 45*, 252–8 (1955).
54. Eckerson, S. H., and Dickson, J. G., *Phytopathology, 13*, 50–1 (1923). (Abs.)
55. Erwin, D. C., *Phytopathology, 42*, 32–5 (1952).
56. Evans, E., and Gottlieb, D., *Phytopathology, 42*, 465–6 (1952). (Abs.)
57. Evans, R. I., *Am. J. Botany, 20*, 255–68 (1933).
58. Evans, R. I., *Am. J. Botany, 24*, 214–18 (1937).
59. Ferguson, J., *Phytopathology, 43*, 586–7 (1953). (Abs.)
60. Ferguson, J., *Pacific Coast Nurseryman, 12*, 15, 48 (1953).
61. Foster, A. A., Cairns, E. F., and Hopper, B., *Phytopathology, 46*, 12 (1956). (Abs.)
62. Gallegly, M. E., and Bishop, C. F., *Plant Disease Reptr., 39*, 914–17 (1955).
63. Garrett, S. D., *Biol. Revs. Cambridge Phil. Soc., 13*, 159–85 (1938).
64. Garrett, S. D., *Root Disease Fungi*, Chronica Botanica Co., Waltham, Mass., 1944.
65. Garrett, S. D., and Mann, H. H., *Ann. Appl. Biol., 35*, 435–42 (1948).
66. Gäumann, E., *Phytopathology, 36*, 624–33 (1946).
67. Gillespie, L. J., and Hurst, L. A., *Soil Sci., 6*, 219–36 (1918).
68. Gopalkrishnan, K. S., and Jump, J. A., *Phytopathology, 42*, 338–9 (1952).
69. Goss, R. W., and Afanasiev, M. M., *Neb. Agr. Expt. Sta. Bull. 317* (1938).
70. Gottlieb, D., *Phytopathology, 42*, 9 (1952). (Abs.)
71. Gottlieb, D., Ammann, A., and Carter, H. E., *Plant Disease Reptr., 39*, 219 (1955).
72. Gottlieb, D., Hassan, H. H., and Linn, M. B., *Phytopathology, 40*, 218–19 (1950).
73. Gray, R., *Plant Physiol., 30* (Suppl.) VI, Sept. 1955.
74. Gregory, K. F., Allen, O. N., Riker, A. J., and Peterson, W. H., *Phytopathology, 42*, 613–22 (1952).
75. Gries, G. A., *Phytopathology, 41*, 15 (1951). (Abs.)
76. Gries, G. A., Horsfall, J. G., and Jacobson, H. G. M., *Phytopathology, 34*, 1001 (1944). (Abs.)
77. Halleck, F. E., and Cochrane, V. W., *Phytopathology, 40*, 715–18 (1950).
78. Halsted, B. D., *N. J. Agr. Expt. Sta. Bull. 112* (1895).
79. Hawkins, L. A., and Harvey, R. B., *J. Agr. Research, 18*, 275–97 (1919).

80. Hebert, T. T., *Plant Disease Reptr.*, *39*, 20–2 (1955).
81. Henry, A. W., *Can. J. Research*, *4*, 69–77 (1931).
82. Henry, B. W., *Phytopathology*, *43*, 81–8 (1953).
83. Hickman, C. J., and English, M. P., *Brit. Mycol. Soc. Trans.*, *34*, 223 36 (1951).
84. Hildebrand, A. A., and West, P. M., *Can. J. Research C19*, 183–98 (1941).
85. Hills, F. J., and Leach, L. D., *Proc. Am. Soc. Sugar Beet Technol.* 549–53 (1952).
86. Hoogerheide, J. C., *Botan. Rev.*, *10*, 599–638 (1944).
87. Hooker, A. L., *Proc. Iowa Acad. Sci.*, *60*, 158–62 (1953).
88. Hooker, W. J., *Plant Disease Reptr.*, *38*, 187–92 (1954).
89. Hooker, W. J., and Peterson, L. E., *Phytopathology*, *42*, 583–91 (1952).
90. Horsfall, J. G., and Dimond, A. E., *Trans. N. Y. Acad. Sci.*, *13*, 338–41 (1951).
91. Horsfall, J. G., and Dimond, A. E., *Ann. Rev. Microbiol.*, *5*, 209–22 (1951)
92. Horsfall, J. G., and Zentmyer, G. A., *Proc. 17th Natl. Shade Tree Conf.*, *17*, 7–15 (1941).
93. Horsfall, J. G., Hollis, J. P., and Jacobson, H. G. M., *Phytopathology*, *44*, 19–24 (1954).
94. Horsfall, J. G., Dimond, A. E., Stoddard, E. M., and Chapman, R. A., *Proc. 2nd Intern. Congr. Crop Protection, 1949.*
95. Houghland, G. V. C., and Cash, L. C., *Plant Disease Reptr.*, *38*, 777–80 (1954).
96. Howard, F. L., *Science*, *94*, 345 (1941).
97. Howard, F. L., Rowell, J. B., and Keil, H. L., *R. I. Agr. Expt. Sta. Bull. 308* (1951).
98. Hutchins, L. M., *23rd Proc. Natl. Shade Tree Conf.*, pp. 17–28, 1947.
99. Johnson, G., and Schaal, L. A., *Science*, *115*, 627–9 (1952).
100. Johnson, J., *Soil Sci.*, *61*, 83–91 (1946).
101. Johnson, L. F., *Phytopathology*, *42*, 468 (1952). (Abs.)
102. Johnson, L. F., *Phytopathology*, *46*, 16 (1956). (Abs.)
103. Jones, L R., and Gilman, J. C., *Wisconsin Agr. Expt. Sta. Research Bull. 38* (1915)
104. Kendrick, J B., Jr., *Phytopathology*, *41*, 20 (1951). (Abs.)
105. Kendrick, J. B., Jr., and Middleton, J. T., *Plant Disease Reptr.*, *38*, 350–3 (1954).
106. Keyworth, W. G., and Dimond, A. E., *Phytopathology*, *42*, 311–15 (1952).
107. Kincaid, R. R., and Gammon, N., Jr., *Plant Disease Reptr.*, *38*, 852–3 (1954)
108. Klotz, L. J., *Science*, *66*, 631–2 (1927).
109. Kommedahl, T., and Young, H. C., *Plant Disease Reptr.*, *40*, 28–9 (1956).
110. Kühn, J., *Biedermanns Zentr.*, *1880*, 859–65.
111. Lachance, R. O., and Perrault, C., *Can. J. Botany*, *31*, 515–21 (1953).
112. Last, F. T., *Ann. Appl. Biol.*, *39*, 557–68 (1952).
113. Leach, L. D., *J. Agr. Research*, *75*, 161–79 (1947).
114. Leach, L. D., and Davey, A. E., *J. Agr. Research*, *64*, 1–18 (1942).
115. Leben, C., and Arny, D. C., *Phytopathology*, *44*, 329–30 (1954).
116. Leukel, R. W., *U. S. Dept. Agr. Yearbook 1953*, pp. 134–45.
117. Linford, M. B., *Phytopathology*, *17*, 133–4 (1927).

118. Linford, M. B., and Vaughan, R. E., *Wisconsin Agr. Ext. Ser. Circ. 188* (1925).
119. Link, K. P., and Walker, J. C., *J. Biol. Chem. 100*, 379–83 (1933).
120. Ludwig, R. A., and Henry, A. W., *Can. J. Research, 21*, 343–50 (1943).
121. Luke, H. H., *Phytopathology, 42*, 286 (1952). (Abs.)
122. Luke, H. H., *Phytopathology, 42*, 469 (1952). (Abs.)
123. Martin, H., *Scientific Principles of Plant Protection*, 3rd ed., Edward Arnold and Co., London, England, 1940.
124. Martin, H., *Can. Dept. Agr., Publ.*, 2nd ed., 1953. (Loose leaf.)
125. Matkin, O. A., and Chandler, P. A., *Calif. Agr. Expt. Sta. Manual*, in press, 1957.
126. McCubbin, W. A., *Plant Quarantine Problem*, Chronica Botanica Co., Waltham, Mass., 1954.
127. McGahen, J. W., *Phytopathology, 41*, 25 (1951). (Abs.)
128. McKay, M. B., *J. Agr. Research, 32*, 437–70 (1926).
129. McKeen C. D., *Can. J. Botany, 32*, 101–15 (1954).
130. McNew, G. L., *U. S. Dept. Agr. Yearbook 1953*, pp. 100–14.
131. McNew, G. L., McCallan, S. E. A., and Miller, P. R., *Boyce Thompson Inst. Plant Research, Professionol Paper*, Reprint 694, 1951.
132. Meredith, C. H., *Phytopathology, 34*, 426–9 (1944).
133. Middleton, J. T., Kendrick, J. B., Jr., and Hall, B. J., *Plant Disease Reptr., 36*, 188–93 (1952).
134. Miles, L. E., *Miss. Agr. Expt. Sta. Tech. Bull. 23* (1936).
135. Mishra, J. N., *Phytopathology, 43*, 338–40 (1953).
136. Mitchell, J. W., Marth, P. C., and Preston, W. H., Jr., *Science, 120*, 263–5 (1954).
137. Moore, W. D., *Phytopathology, 39*, 920–7 (1949).
138. Moore, W. D., and Conover, R. A., *Plant Disease Reptr., 39*, 103–5 (1955).
139. Morton, D. J., and Stroube, W. H., *Phytopathology, 45*, 417–20 (1955).
140. Munger, H. M., and Newhall, A. G., *Phytopathology, 43*, 254–9 (1953).
141. Munnecke, D. E., and Ferguson, J., *Phytopathology, 43*, 375–7 (1953).
142. Neal, D. C., *Ann. Missouri Botan. Garden, 14*, 359–424 (1927).
143. Newburgh, R. W., and Cheldelin, V. H., *Plant Disease Reptr., 39*, 684 (1955).
144. Newhall, A. G., *Soil Sci., 61*, 67–82 (1946).
145. Newhall, A. G., *Botan. Rev., 21*, 189–250 (1955).
146. Ornamental Crops Subcommittee, Committee on Seed and Plant Material Certification, The American Phytopathological Society, *Plant Disease Reptr. Suppl., 238* (1956).
147. Orton, W. A., *U. S. Dept. Agr. Yearbook 1908*, 453–64 (1909).
148. Oswald, J. W., and Lorenz, O. A., *Phytopathology, 46*, 22 (1956). (Abs.)
149. Oswald, J. W., and Wright, D. W., *Phytopathology, 41*, 29 (1951). (Abs.)
150. Pryor, D. E., *J. Agr. Research, 61*, 149–60 (1940).
151. Reinking, O. A., and Manns, M. M., *Z. Parasitenk., 6*, 23–75 (1933).
152. Richardson, H. H., and Johnson, A. C., *U. S. Dept. Agr. Tech. Bull. 853* (1943).
153. Rochlin, E., *Phytopathology, 5*, 381–406 (1932).
154. Roistacher, C. N., *Phytopathology, 41*, 943 (1951). (Abs.)
155. Roistacher, C. N., Baker, K. F., and Bald, J. G., *Hilgardia*, in press, 1957.

156. Roth, L. F., and Riker, A. J., *J. Agr. Research, 67,* 273–93 (1943).
157. Rudd, W. N., *Am. Florist, 9,* 171 (1893).
158. Rushdi, M., and Jeffers, W. F., *Phytopathology, 46,* 88–90 (1956).
159. Sanford, G. B., *Can. J. Research, C19,* 1–8 (1941).
160. Sanford, G. B., *Soil Sci., 61,* 9–21 (1946).
161. Sanford, G. B., *Can. J. Botany, 30,* 652–64 (1952).
162. Sanford, G. B., and Broadfoot, W. C., *Sci. Agr., 11,* 512–28 (1931).
163. Schaal, L. A., and Johnson, G., *Phytopathology, 45,* 626–8 (1955).
164. Schroeder, R. A., and Albrecht, W. A., *Soil Sci., 53,* 481–8 (1942).
165. Smith, P. G., and Walker, J. C., *J. Agr. Research, 63,* 1–20 (1941).
166. Smith, R. M., Peterson, W. H., and McCoy, Elizabeth, *Antibiotics & Chemotherapy, 4,* 962–70 (1954).
167. Smoot, J. J., *Phytopathology, 41,* 565 (1951). (Abs.)
168. Sowell, G., Jr., *Phytopathology, 46,* 27 (1956). (Abs.)
169. Stahmann, M. A., Link, K. P., and Walker, J. C., *J. Agr. Research, 67,* 49–63 (1943).
170. Stallings, J. H., *Bacteriol. Revs., 18,* 131–46 (1954).
171. Stark, F. L., Jr., *Cornell Univ. Agr. Expt. Sta. Mem. 278,* Aug. 1948.
172. Stevenson, F. J., and Akeley, R. V., *Phytopathology, 43,* 245–53 (1953).
173. Stevenson, F. J., and Jones, H. A., *U. S. Dept. Agr. Yearbook 1953,* pp. 192–216.
174. Stoddard, E. M., *Phytopathology, 41,* 858 (1951).
175. Stoddard, E. M., and Dimond, A. E., *Botan. Rev., 15,* 345–76 (1949).
176. Stoddard, E. M., and Dimond, A. E., *Phytopathology, 41,* 337–40 (1951).
177. Stover, R. H., *Phytopathology, 43,* 499–504 (1953).
178. Stover, R. H., *Can. J. Botany, 31,* 693–7 (1953).
179. Stover, R. H., *Soil Sci., 77,* 401–14 (1954).
180. Stover, R. H., *Soil Sci., 80,* 397–412 (1955).
181. Stover, R. H., Thornton, N. C., and Dunlap, V. C., *Phytopathology, 42,* 476 (1952). (Abs.)
182. Stover, R. H., Thornton, N. C., and Dunlap, V. C., *Soil Sci., 76,* 225–38 (1953).
183. Streets, R. B., *Phytopathology, 41,* 944 (1951). (Abs.)
184. Taubenhaus, J. J., and Ezekiel, W. N., *Am. J. Botany, 23,* 10–12 (1936).
185. Thornton, H. G., *Rept. Rothamst. Expt. Sta., 1952,* 58–64 (1953).
186. Timonin, M. I., *Can. J. Research C18,* 444–56 (1940).
187. Torgeson, D. C., *Contribs. Boyce Thompson Inst., 17,* 359–73 (1954).
188. Tyner, L. E., *Phytopathology, 43,* 313–16 (1953).
189. Valleau, W. D., Johnson, E. M., and Irvine, J. W., *Ky. Agr. Expt. Sta. Bull 592* (1952).
190. Vaughn, J. R., *Phytopathology, 41,* 36 (1951). (Abs.)
191. Vaughn, J. R., and Klomparens, W., *Phytopathology, 42,* 22 (1952). (Abs.)
192. Waggoner, P. E., and Shaw, R. H., *Phytopathology, 43,* 317–18 (1953).
193. Waggoner, P. E., and Taylor, G. S., *Am. Potato J., 32,* 168–72 (1955).
194. Waksman, S. A., *Soil Sci., 3,* 565–89 (1917).
195. Waksman, S. A., *Soil Sci., 14,* 61–79 (1922).
196. Waksman, S. A., *Bacteriol. Revs., 5,* 231–91 (1941).

197. Walker, J. C., *Phytopathology, 10*, 323–7 (1920).
198. Walker, J. C., *Phytopathology, 16*, 697–710 (1926).
199. Walker, J. C., *Botan. Rev., 7*, 458–506 (1941).
200. Walker, J. C., *Soil Sci., 61*, 47–54 (1946).
201. Walker, J. C., *Plant Pathology*, 1st ed., McGraw-Hill Book Co., Inc., New York, 1950.
202. Walker, J. C., and Foster, R. E., *Am. J. Botany, 33*, 259–64 (1946).
203. Walker, J. C., and Hare, W. W., *Wis. Agr. Expt. Sta. Research Bull. 145* (1943).
204. Walker, J. C., Link, K. P., and Angell, H. R., *Proc. Natl. Acad. Sci. U.S., 15*, 845–50 (1929).
205. Walker, J. C., Gallegly, M. E., Jr., Bloom, J. R., and Scheffer, R. P., *Am. J. Botany, 41*, 760–2 (1954).
206. Wallen, V. R., and Skolko, A. J., *Plant Disease Reptr., 37*, 421–4 (1953).
207. Weindling, R., *Phytopathology, 22*, 837–45 (1932).
208. Weindling, R., *Phytopathology, 24*, 1153–79 (1934).
209. Weindling, R., *Botan. Rev., 4*, 475–96 (1938).
210. Weindling, R., *Soil Sci., 61*, 23–30 (1946).
211. Weindling, R., and Emerson, O. H., *Phytopathology, 26*, 1068–70 (1936).
212. Weindling, R., and Fawcett, H. S., *Hilgardia, 10*, 1–16 (1936).
213. Wellman, F. L., *U. S. Dept. Agr. Tech. Bull. 181* (1930).
214. West, P. M., and Hildebrand, A. A., *Can. J. Research, 19*, 199–210 (1941).
215. Wilhelm, S., *Phytopathology, 40*, 970 (1950). (Abs.)
216. Wilhelm, S., *Phytopathology, 41*, 684–90 (1951).
217. Wilhelm, S., *Phytopathology, 41*, 944 (1951). (Abs.)
218. Wilhelm, S., *Phytopathology, 45*, 180–1 (1955).
219. Wilhelm, S., *Phytopathology, 45*, 696 (1955). (Abs.)
220. Wilhelm, S., and Ferguson, J., *Phytopathology, 43*, 593–6 (1953).
221. Wingard, S. A., *Botan. Rev., 7*, 59–109 (1941).
222. Wingard, S., *U. S. Dept. Agr. Yearbook 1953*, pp. 165–73.
223. Wood, R. K. S., and Tveit, M., *Botan. Rev., 21*, 441–92 (1955).
224. Young, V. H., and Tharp, W. H., *Arkansas Agr. Expt. Sta. Bull. 410* (1941).
225. Zak, B., *Phytopathology, 44*, 511 (1954). (Abs.)
226. Zeller, S. M., *Oregon Agr. Expt. Sta. Bull. 344* (1936).
227. Zentmyer, G. A., *Phytopathology, 32*, 20 (1942). (Abs.)
228. Zentmyer, G. A., *U. S. Dept. Agr. Yearbook 1953*, pp. 875–81.
229. Zentmyer, G. A., *Phytopathology, 44*, 511–12 (1954). (Abs.)
230. Zentmyer, G. A., *Phytopathology, 45*, 398–404 (1955).
231. Zentmyer, G. A., and Bingham, F. T., *Phytopathology, 46*, 121–4 (1956).
232. Zentmyer, G. A., and Horsfall, J. G., *Phytopathology, 33*, 16–17 (1943). (Abs.)
233. Zentmyer, G. A., and Kendrick, J. B., Jr., *Phytopathology, 39*, 864 (1949). (Abs.)
234. Zentmyer, G. A., and Klotz, L. J., *Phytopathology, 39*, 26–7 (1949). (Abs.)
235. Zentmyer, G. A., and Richards, S. J., *Phytopathology, 42*, 35–7 (1952).
236. Zentmyer, G. A., Horsfall, J. G., and Wallace, P. P., *Conn. Agr. Expt. Sta. Bull. 498* (1946).

# REPELLENTS FOR BITING ARTHROPODS

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## I. Introduction

The origins of repellents to keep insects away from man's person, shelter, and food are lost in antiquity. Smokes from domestic fires or from special substances, plants hung in dwellings or rubbed on the body (either fresh or as brews), oils, pitches, tars, and various earths applied to the body, have been used through the ages as insect repellents (15). Indeed, some of these are still in use. In 1923, Howard (54) recommended several essential oils as mosquito repellents, one of which was oil of citronella, which had been in use for several years and remained the repellent of choice for the next 15 or 20 years. Emphasis on chemicals other than plant products was stimulated by the work of Granett (40). During World War II, many thousands of compounds were synthesized and tested under military auspices, since the need to protect troops against disease vectors was acute. Today, the most effective arthropod repellents in use are synthetic organic chemicals, although an occasional formulation of oil of citronella or other essential oil is encountered. In recent years research on arthropod repellents for protection of humans has been directed not only against mosquitoes, but also against other biting flies (black flies, horse flies, deer flies, sand flies, horn flies), chigger mites, ticks, and fleas. Repellents for protection of livestock are directed primarily against the biting flies.

Since the greatest emphasis in the search for repellents has been for the individual protection of humans, performance characteristics of the ideal repellent include criteria based on the application of the repellent to the skin and on necessary avoidance of irritation or adverse esthetic appearance (15,41). Such criteria assume to a great extent that the ideal repellent must be applied to large areas of skin or clothing and, therefore, reflect only limitations which must be placed on present repellents since all are used in this manner. If criteria for a truly ideal repellent based on ultimate desirability and performance are to be stated, the following requirements are suggested: (a) Complete protection of the individual from insect feeding for as long as possible under any conditions of infestation, (b) maximum effectiveness in minimum quantity (great inherent repellency), (c) lack of necessity of being applied to areas to be protected, and (d) lack of undesirable odor. Criteria such as these, except for (d), make no reference to the method of application or nature of the repellent. Other criteria, such as cheapness and lack of toxicity, are obviously desirable but are problems of development which follow attempts to achieve an unrestricted ideal. It will be apparent from the following discussion that the ideals of Granett (41) and Dethier (15), or those proposed above, have not been fully achieved. However, more progress has been made since 1935 than was made prior to that date.

For further discussion of natural and synthetic insect attractants and repellents, and for a discussion of their mode of action, see Dethier (15,20). A more fundamental review of insect chemoreception is given by Dethier and Chadwick (22) and Hodgson (51).

## II. Methods of Testing Repellents

One encounters difficulty in attempting to determine effectiveness of repellents through a study of the literature because of a lack of standardized testing procedures, characterized by diverse criteria for effectiveness and the use of different test species. The choice of the method of testing depends, of course, on the amount of specific information needed. One cannot doubt a subjective assertion that each of several compounds repels mosquitoes for several hours, but the information is of little importance unless some comparison of relative effectiveness of such compounds can be made. One of the first attempts to standardize the procedures for quantitatively assessing the repellency of various candidate compounds was made by Granett (40) and later modified by him (42). The principle of his technique does not differ from routine application to the skin. The importance of the work is that the amount of compound applied, the area to which it was applied, and the criterion of effectiveness (time to the first bite) were defined so that discrete expressions of relative performance could be made.

Although McCulloch and Waterhouse (73) suggest that human arms differ from legs in attractiveness to mosquitoes, Travis *et al.* (101), in field tests, could not show a significant difference in repellent time between material applied to arms and legs. Kasman *et al.* (59) compared a method of testing the repellency of compounds applied to the backs of guinea pigs then spread with the finger, with the same compounds applied to the human arm. Values obtained from both procedures were approximately the same. Eddy and McGregor (26) obtained comparable results from compounds sprayed on cows and on white mice in repellency and toxicity tests on stable flies. Wasicky *et al.* (108) used canaries and guinea pigs while Wiesmann and Lotmar (109) and Healy (50) used white mice in testing repellents. Starnes and Granett (95) tested stable flies in a cage, whose end was covered with a cloth treated with repellent and held against the shaven belly of a rabbit. It is apparent that animals other than man can be used effectively as attractants against which to base repellency tests. Although such problems as the number of subjects required and unsuspected dermatological effects, which arise from using human subjects in initial screening of compounds, may be obviated by the use of other laboratory animals, many variables indigenous to the host and to the repellent still exist, such as absorption by the skin, volatility, modification of the compound and differences between individuals in attractiveness. As aptly pointed out by Dethier (20) "Yet, like tests on man, these can serve only the purpose of quick gross screening." In later years, the methods for initial testing of candidate compounds against biting arthropods have been somewhat standardized. Methods vary, however, according to the laboratory and the species of insect involved. In general, compounds applied to skin and to cloth are tested against mosquitoes and other biting flies, whereas the testing of compounds against fleas, ticks, chigger mites, and lice is done almost exclusively with compounds applied to cloth. To avoid a tiresome reiteration of techniques for each test species, only the more important methods and major variations in them will be discussed.

King (61) described a method for testing repellency to lice and chiggers by confining them on treated cloth patches. A compound was rated according to speed of paralysis ["stopping time" of Snyder and Morton (93)] and ability to withstand washing. The method of applying the material to the cloth patch varies according to the solubility of the compound; usually acetone is the solvent. A compound was considered effective if paralysis of lice was complete within one hour and was given a rating of 4. If paralysis took place within 15 minutes, the compound was given a rating of 4a. If the compound still showed promise, it was rinsed in cold water and retested after 48 hours. The patches were subjected to standardized washings with soap and hot water until the treatment failed to paralyze the chiggers within 15 minutes. As pointed out by King (61), materials selected by these tests are fast-acting toxicants rather than true repellents.

Brody (7) inserted ticks in a wire cage which covered a treated ear of a cow. Repellency was determined by the number of ticks dead, attached, or engorged. Although Smith and Gouck (90) were unsuccessful in evaluating repellents in the field by allowing ticks to cross bands of treated cloth or skin, Granett and Sacktor (48) report a method in which a count is made of the number of ticks that crawl from the untreated center of a piece of filter paper across a band of chemical sprayed around the outer border of the paper. For the initial phase of screening of tick repellents, King (61) determined the number of ticks crossing the midline of a treated 4-inch square cloth patch which was secured to a small paddle. Class 4 compounds were required to be 80% repellent (when measured against untreated cloth) for more than 10 days. A test was described by Smith and Gouck (90) and King (61) wherein human subjects wore men's cotton socks treated from the ankle to the top. The subjects sat with their feet in a pen infested with ticks. The number that crawled to the top of the sock was counted and the compound rated as for the patch test.

Linduska *et al.* (66) developed a method of screening flea repellents which consisted of waving treated and untreated cloth patches over a pen within jumping distance of the fleas. When several had alighted on the patches, the number of fleas remaining on each for a minute was noted. Percent repellency was calculated from the formula $100\ (a\text{-}b)/a$, where $a$ is the number of insects on the untreated cloth and $b$ the number on the treated cloth. A class 4 compound must be 80% repellent for more than 10 days. As in the case of tick repellents, a test for flea repellents is carried out by having subjects expose treated cotton stockings in a pen of fleas and then counting the number of fleas that reached the top of the stocking. The criterion for effective compounds is 90% repellency for more than 10 days (61).

In addition to tests on arms of human subjects (61), several authors propose methods using small laboratory animals (such as canaries, mice, rabbits) for screening of repellents for biting flies. Pearson (77) tried to take into consideration differences in susceptibility to fly attack among cows by taking hourly counts of flies on each cow during the pretreatment days. He arranged the cows in groups of five according to individual susceptibility during the testing of repellents. Mailen and Fenton (70) describe a "half-cow" method whereby two compounds can be tested simultaneously. Granett *et al.* (47) used the "half-cow" method, expressing the data in percent repellency after correcting the normal counts for inherent susceptibility of the cows to insect attack. Fryer *et al.* (31) found a large number of variables in the fly repellency tests on cattle. Periodic counts of flies on cows indicate a Poisson-like distribution which does not approach a normal distribution. A reciprocal square root transformation of the data was suggested, followed by an analysis of variance. Fryer *et al.* (30) compared "half-cow" and "whole-cow" methods of conducting field tests. By adequate experimental design, it was found that the "whole-cow" test was superior. Other suggestions were to use a log transformation of data followed by analysis by T-test or F-test. Using a technique of repellent screening on white mice, Healy (50) advocates allocating more mice for each treated group than the controls since the latter receive greater weight with the higher attack rate. Estimation of parameters was by weighted least squares. Wadley (107) suggests an incomplete-block design adapted to paired tests of mosquito repellents, called "unpaired tests" by Travis *et al.* (102), to gain a 90% efficiency over completely randomized blocks, if the gain is available. Although experimental error is slightly higher in the incomplete-block design than in the direct comparison of test and standard materials, the error for incomplete-block design is definitely lower than that for the comparison of two test substances with the standard.

Since the preliminary work by Granett (40), little has been done to control the many variables inherent in screening repellents to biting flies, especially those variations between test hosts and between individual insects or populations of them. Among various workers the amount of repellent applied and the area covered varied considerably [$^1/_4$ teaspoon/arm, $^3/_8$ teaspoon/leg (99); 1 cc./arm, 2 cc./leg (40); 1 cc./100 sq. in. (45); 1 ml./arm, 1.5 ml./leg (61); 0.5 to 0.8 cc./hand and arm, 3 cc./leg (73); 1 quart/horse (38); 4 drops/arm, 6 drops/leg (58)]. Some compounds were tested immediately after application, others were applied twenty-four hours before being tested.

The criteria of the loss of effectiveness of repellent compounds are diversified. Biting rate was used by Healy (50), length of time to first bite by Granett (40) and many others, time to second bite (107), time to third bite (14), time to fifth bite (66), time until 50% of the population had fed (95), total numbers of flies at a given time (77). Length of time of exposure ranged from continuous exposure with counts every hour (77) to a five-minute exposure every 20 minutes (101), a three-minute exposure every 30 minutes (61), and a two-minute exposure every 15 to 30 minutes (40). At first, compounds were tested individually (40). The technique was later changed so that two compounds were tested simultaneously on the same host since relative effectiveness was considered to be of prime importance (42). Usually an unknown compound was tested on one arm against a standard repellent (such as oil of citronella or dimethyl phthalate) on the other arm. Later (102,107) the technique was modified so that the standard was run randomly in a series of compounds, but not more than any other compound (incomplete-block design). The comparison with the standard is made statistically. Both "paired" and "unpaired" (or incomplete block) were compared by Travis *et al.* (102).

Emphasis has been placed on the ability of compounds to withstand wiping or rubbing of the skin (10), rinsing the skin with water (61), and perspiration (79). Practical tests are run on biting fly repellents to determine their effectiveness and duration of activity when applied to cloth (61,34,35).

A comparison of results of tests on repellents conducted in separate laboratories still is very difficult. Ignoring for the moment the variables in the insects, great divergences in the methods of testing still exist. Many laboratories use undiluted compounds while others test the candidate repellents in various solvents. A standard empirical screening method, acceptable to all laboratories, is urgently needed. In addition, better quantitative methods for measuring inherent repellency are needed in order to obtain a better understanding of the mode of action of repellents and to provide more exact data for studies on chemical structure and repellent activity. Although olfactometers measure inherent repellency (17), they are not extensively used in repellent studies (10,23,65,78,86) since they involve laborious techniques. Also, results from olfactometer studies generally are not considered to be directly translatable into field conditions.

Certain other techniques used in studying chemoreception appear promising to the study of repellents. It would seem feasible to consider a modification of the techniques of Dethier and Chadwick (21), which deal with thresholds of individuals rather than relying on the response of a large population. In view of the electrophysiological work of Hodgson *et al.* (51,52) with a single chemoreceptor unit, the study of action potentials from olfactory receptors has been given added credulity. Previous attempts in this area have failed (9,22). Although Roys (84) was able to record bursts of activity in antennal nerve preparations produced by vapors of benzene and toluene, these were regarded as involving no specific receptor mechanisms.

## III. Chemical Repellents

There are not more than a dozen compounds which are in widespread use as repellents for biting arthropods. Until the last ten years, comparatively little was published about the chemical constitution of repellents or about the repellent action of organic chemicals. Fortunately, compilations (61,75,81,102) have appeared since then in the literature. The most fertile source of information concerning repellency of chemicals to lice, fleas, chigger mites, ticks, and mosquitoes was presented by King (61). At times these compilations can be deceiving, since most of them do not refer to any properties of the chemicals other than repellency. Although the pharmacology of repellent chemicals is determined before extensive laboratory and field tests can be run (24,76), much of this information is never reported in the literature. There have been some instances of deleterious effects attributed to various skin-applied repellents (53,67).

The data presented in Table I were taken from the open literature. As stated in the above section, methods of testing vary so greatly that the task of assessing the screened compounds was prodigious. The criteria used in selecting compounds to be cited were empirical.

## TABLE I

Chemical Repellents Found Effective in Laboratory or Field Tests

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| Acetamide, *N*-cyclohexyl-alpha-butoxy- | *Aedes* spp. | C | 61 |
| | *Trombicula* spp. | C | 61 |
| Acetamide, *N*-cyclohexyl-2-butoxy-ethoxy- | *Aedes aegypti* | C | 61 |
| | *Anopheles quadrimaculatus* | S | 61 |
| Acetanilide, *N*-*n*-butyl- | *Pediculus humanus* | C | 61 |
| | *Aedes aegypti* | S&C | 61 |
| | *Anopheles quadrimaculatus* | S | 61 |
| | *Trombicula* spp. | C | 6,61 |
| | *Amblyomma americanum* | C | 61 |
| | *Xenopsylla cheopis* | C | 6,61 |
| Acetanilide, *N*-*n*-propyl- | *Trombicula* spp. | C | 61 |
| | *Aedes aegypti* | C | 61 |
| | *Anopheles quadrimaculatus* | C | 61 |
| Acetic acid, 2-phenoxyethyl ester | *Trombicula* spp. | C | 61 |
| Acetoacetic acid, 2,2-thiodiethyl ester | Black flies | S | 14 |
| Acetophenone, *p*-methoxy- | *Anopheles quadrimaculatus* | S | 61 |
| | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Pediculus humanus* | C | 61 |
| | *Stomoxys calcitrans* | S | 105 |
| Adipic acid, di-*n*-butyl ester | *Dermacentor variabilis* | S&C | 43,44,61 |
| Adipic acid, di-*n*-propyl ester | *Dermacentor variabilis* | C | 43,61 |
| | *Trombicula* spp. | C | 61 |
| Benzamide, *o*-chloro-*N*,*N*-diethyl- | *Aedes aegypti* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Aedes communis* | S | 1 |
| | *Aedes punctor* | S | 1 |
| | *Aedes excrucians* | S | 1 |
| | *Aedes fitchii* | S | 1 |
| | *Aedes stimulans* | S | 1 |
| Benzamide, *o*-ethoxy-*N*,*N*-diethyl- | *Aedes communis* | S | 1 |
| | *Aedes punctor* | S | 1 |
| | *Aedes excrucians* | S | 1 |
| | *Aedes fitchii* | S | 1 |
| | *Aedes stimulans* | S | 1 |
| | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 39 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |

TABLE I (*Continued*)

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| | *Anopheles albimanus* | S | 34 |
| | *Anopheles strodei* | S | 34 |
| | *Anopheles taeniorhynchus* | S | 35 |
| | *Aedes sollicitans* | S | 35 |
| | *Psorophora confinis* | S | 35 |
| Benzoic acid, 2-chlorophenyl ester | *Trombicula* spp. | C | 61 |
| Benzoic acid, benzyl ester | *Trombicula* spp. | C | 61 |
| Benzyl ether | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Aedes aegypti* | S | 61 |
| | *Culicoides* spp. | S | 99 |
| Bicyclo (2.2.1)-5-heptene-2,3-dicarboxylic acid, *cis*-, dimethyl ester (dimethyl carbate) | *Aedes aegypti* | S&C | 61 |
| | *Anopheles quadrimaculatus* | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Culicoides* spp. | S | 100 |
| | *Simulium* spp. | S | 100 |
| Butadiene, furfural copolymer | *Stomoxys calcitrans* | C | 36 |
| 3-Buten-1-ol, 1-(*o*-methoxyphenyl)- | *Aedes aegypti* | C | 61 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |
| | *Anopheles albimanus* | S | 34 |
| | *Anopheles strodei* | S | 34 |
| Chloroethylamide, trichloroacetyl- | Culicine mosq. | S | 62 |
| Cinnamic acid, *n*-propyl ester | *Trombicula* spp. | C | 61 |
| Citronellal | *Aedes aegypti* | S | 10 |
| | *Anopheles punctulatus farauti* | S | 73 |
| Cyclohexanol, 2-cyclohexyl- | *Trombicula* spp. | C | 61 |
| | *Aedes aegypti* | C&S | 61,58 |
| | *Amblyomma americanum* | C | 61 |
| 3,5-Decanediol, 2-methyl- | *Amblyomma americanum* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Anopheles quadrimaculatus* | C | 61 |
| 1,2-Cyclohexane dicarboxylic acid, diethyl ester | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| Ethanol, 2-(2-butoxyethoxy-), acetate | *Trombicula* spp. | C | 61 |
| | *Aedes aegypti* | C | 61 |

*Table continued*

TABLE I (*Continued*)

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| | *Eusimulium pecuarum* | S | 104 |
| | *Prosimulium hirtipes* | S | 104 |
| | *Simulium venustum* | S | 104 |
| Ethanol, 2(2-(2-ethylhexyloxy)-ethoxy)- | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Aedes aegypti* | S&C | 61 |
| | *Aedes prionips* | C | 3 |
| | *Aedes excrucians* | C | 3 |
| | *Aedes communis* | C | 3 |
| Ethanol, 2-phenoxy, acetate | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| Furfural, 2,3,4,5-bis-(2-butenylene)-tetrahydro- | *Stomoxys calcitrans* | C | 56,57 |
| Furfuryl alcohol,2,3,4,5-bis-(2-butenylene)-tetrahydro- | *Stomoxys calcitrans* | C | 56,57 |
| 1,3-Hexanediol, 2-ethyl- (Rutgers 612) | *Trombicula* spp. | C | 61,45,100 |
| | *Aedes aegypti* | C | 61 |
| | Anopheline mosq. | S&C | 45 |
| | Culicine mosq. | S&C | 45 |
| | *Stomoxys calcitrans* | S&C | 45 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |
| | *Culicoides* spp. | S&C | 45,100 |
| | *Aedes* spp. | S | 105,106 |
| | Mosquitoes | C | 98 |
| | | S | 100,105 |
| | *Simulium* spp. | S&C | 14,100,104 |
| Hydracrylic acid, $\beta$-phenyl ethyl ester | *Aedes aegypti* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | S&C | 61 |
| Isobutyric acid, $\alpha$-hydroxy-, phenethyl ester | *Aedes aegypti* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Culicoides* spp. | S | 99 |
| Isobutyric acid, 2-phenoxyethyl ester | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61 |
| *d,l*-Malic acid, dibutyl ester | *Trombicula* spp. | C | 61 |
| | *Aedes aegypti* | S&C | 61 |

TABLE I (*Continued*)

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| | *Eusimulium pecuarum* | S | 104 |
| | *Culicioides* spp. | S | 99 |
| Mandelic acid, amyl ester | *Aedes aegypti* | S&C | 61 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |
| | *Anopheles albimanus* | S | 34 |
| | *Anopheles strodei* | S | 34 |
| | *Amblyomma americanum* | C | 34 |
| | *Aedes communis* | C | 1 |
| | *Aedes punctor* | C | 1 |
| | *Aedes excrucians* | C | 1 |
| | *Aedes fitchii* | C | 1 |
| | *Aedes stimulans* | C | 1 |
| Mandelic acid, hexyl ester | *Aedes aegypti* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61,88 |
| | *Dermacentor variabilis* | C | 44 |
| | Mosquitoes | C | 97 |
| | *Simulium* spp. | C | 97 |
| | *Culicoides* spp. | C | 97 |
| Mandelic acid, *n*-propyl ester | *Aedes aegypti* | C | 61 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |
| | *Anopheles albimanus* | S | 34 |
| | *Anopheles strodei* | S | 34 |
| Mercaptopolyoxyethylene, *tert*-dodecyl- | *Stomoxys calcitrans* | C | 36 |
| α-Naphthol (1-naphthol) | *Aedes aegypti* | C | 61 |
| | *Anopheles quadrimaculatus* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61 |
| β-Naphthol (2-naphthol) | *Aedes aegypti* | C | 61 |
| | *Anopheles quadrimaculatus* | C | 61 |
| | *Aedes* spp. | C | 60 |

*Table continued*

TABLE I (*Continued*)

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| Phenol, 2,3,4,6-tetrachloro- | *Aedes aegypti* | C | 61 |
| | *Aedes* spp. | C | 60 |
| | *Trombicula* spp. | C | 61 |
| | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61 |
| Phenol, 2,4,6-trichloro- | *Trombicula* spp. | C | 61 |
| Phenoxathiin | *Trombicula* spp. | C | 61 |
| Phthalic acid, dibutyl ester | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| Phthalic acid, diethyl ester | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| Phthalic acid, dimethyl ester | *Aedes aegypti* | S&C | 61,73 |
| | *Anopheles quadrimaculatus* | S | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Dermacentor variabilis* | C | 43 |
| | *Aedes* spp. | S&C | 60,88,105,106 |
| | *Eusimulium pecuorum* | S | 104 |
| | *Prosimulium hirtipes* | S | 104 |
| | *Simulium venustum* | S | 104 |
| | *Simulium* spp. | S | 14,60 |
| | *Chrysops silacea* | S | 13 |
| | *Chrysops dimidiata* | S | 13 |
| | *Culicoides tristriatulus* | S | 3 |
| | *Anopheles punctulatus farauti* | S | 73 |
| | *Aedes vigilax* | S | 73 |
| | *Aedes flavescens* | S | 4 |
| | *Aedes excrucians* | S | 4 |
| | *Aedes communis* | S | 4 |
| | *Aedes prionips* | S | 4 |
| | *Amblyomma americanum* | C | 61 |
| Phthalimide, *N-sec*-butyl- | *Aedes aegypti* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61 |
| | *Culicoides* spp. | S | 99 |
| Piperonyl alcohol, $\alpha$-*tert*-butyl- | *Aedes aegypti* | C | 61 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |
| | *Anopheles albimanus* | S | 34 |
| | *Anopheles strodei* | S | 34 |

TABLE I (*Continued*)

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| Polypropyleneglycol, butoxy- (M.W. 800) | *Stomoxys calcitrans* | S | 46,47 |
| | *Siphona irritans* | S | 46 |
| | *Tabanus* spp. | S | 46 |
| 1,3-Propanediol,2-ethyl-2-butyl- | *Aedes aegypti* | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Aedes taeniorhyncus* | S | 89 |
| | *Aedes sollicitans* | S | 89 |
| 1-Propanol,2-amino-3-isobornyloxy-2-methyl- | *Aedes aegypti* | S&C | 61 |
| | *Anopheles quadrimaculatus* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Chrysops discalis* | S | 82 |
| Propionamide, $\alpha$-,$\alpha$-,$\beta$-trichloro- | *Aedes aegypti* | C | 61 |
| | *Anopheles quadrimaculatus* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| 1,5-Pentanediol, dipropionate (propionic acid, diester with 1,5-pentanediol) | *Trombicula* spp. | C | 61 |
| Propiophenone oxime | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| 2H-Pyran-6-carboxylic acid,3,4-dihydro-2,2-dimethyl-4-oxo-, butyl ester (Indalone) | *Aedes aegypti* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Dermacentor variabilis* | C | 43 |
| | *Glossina palpalis* | S | 27 |
| | *Eusimulium pecuarum* | S | 104 |
| | *Prosimulium hirtipes* | S | 104 |
| | *Simulium venustum* | S | 104 |
| | *Aedes taeniorhynchus* | C | 89 |
| | *Aedes sollicitans* | C | 89 |
| | *Aedes* spp. | S | 106 |
| | *Stomoxys calcitrans* | S | 103 |
| Pyridine-2,5-dicarboxylic acid, diethyl ester | *Stomoxys calcitrans* | C | 36 |
| Pyridine-2,5-dicarboxylic acid, dipropyl ester | *Stomoxys calcitrans* | C | 36 |
| Salicylic acid, 2-thenyl ester | *Trombicula* spp. | C | 12 |
| Succinamic acid, *N*,*N*-diethyl propyl ester | *Aedes aegypti* | S&C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Amblyomma americanum* | C | 61 |
| | *Simulium* spp. | S | 14,97 |
| | *Culicoides* spp. | S | 97 |
| | *Aedes taeniorhyncus* | C | 89 |
| | *Aedes sollicitans* | C | 89 |

*Table continued*

TABLE I (*Continued*)

| Chemical | Species | Use[a] | Ref. |
|---|---|---|---|
| | *Aedes flavescens* | S | 4 |
| | *Aedes excrucians* | S | 1,4 |
| | *Aedes communis* | S | 1,4 |
| | *Aedes prionips* | S | 4 |
| | *Aedes punctor* | S | 1 |
| | *Aedes fitchii* | S | 1 |
| | *Aedes stimulans* | S | 1 |
| Succinimide, *N*-amyl- | *Aedes aegypti* | S&C | 61 |
| | *Anopheles quadrimaculatus* | S | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Pediculus humanus* | C | 61 |
| | *Glossina palpalis* | S | 27 |
| | *Culicoides* spp. | C | 99 |
| Thiocyanic acid, *p*-dimethylamine, phenyl ester | *Amblyomma americanum* | C | 61 |
| | *Trombicula* spp. | C | 61 |
| | *Ctenocephalides felis* | C | 61 |
| | *Xenopsylla cheopis* | C | 61 |
| *m*-Toluamide, *N*,*N*-diethyl ester | *Aedes aegypti* | C&S | 35,61 |
| | *Aedes communis* | S | 1 |
| | *Aedes punctor* | S | 1 |
| | *Aedes excrucians* | S | 1 |
| | *Aedes fitchii* | S | 1 |
| | *Aedes stimulans* | S | 1 |
| | *Amblyomma americanum* | C | 39 |
| | *Mansonia titillans* | S | 34 |
| | *Mansonia indubitans* | S | 34 |
| | *Mansonia fasciolata* | S | 34 |
| | *Mansonia nigricans* | S | 34 |
| | *Anopheles albimanus* | S | 34 |
| | *Anopheles strodei* | S | 34 |
| | *Aedes taeniorhynchus* | S | 35 |
| | *Aedes sollicitans* | S | 35 |
| | *Psorophora confinis* | S | 35 |
| | *Anopheles quadrimaculatus* | S | 35 |
| Undecenoic acid | *Aedes communis* | C | 1 |
| | *Aedes punctor* | C | 1 |
| | *Aedes excrucians* | C | 1 |
| | *Aedes fitchii* | C | 1 |
| | *Aedes stimulans* | C | 1 |

[a] C denotes impregnation into cloth, S denotes application to skin.

Table I lists compounds which had been screened as candidate repellents and had been stated to be effective. The compilations of Travis *et al.* (102) and King (61) were not consulted at this point since many of the compounds were never tested further. Compounds stated in the literature to be toxic to the skin were not listed. However, it is probable that certain compounds cited in this table should be eliminated for reasons of their pharmacology, but information on this point is obscure. If a compound was considered effective before 1954, this compound was checked in the work of King (61). If the compound in question was given a class rating of 4 or above (4a) in that report, it was listed in Table I with the species of insects it was reported to repel. For compounds whose activities were previously reported but were summarized in King (61), only that publication is cited. If a compound was not in King (61) or was published later than 1954, only those compounds are listed which were reported to be definitely superior to standard repellents.

There are several chemicals (such as pyrethrum, derris, nicotine) which are primarily insecticides, but also possess repellent properties (15,38,55,83). Dethier (20) has a good discussion of the confusion in the literature on the repellency and/or attractancy of DDT and various moth repellents. As previously indicated, the so-called tick or chigger repellents are, in fact, toxicants, although they may be repellent to mosquitoes or other biting flies.

In 1923, Howard (54) tested the repellent mixtures available then and concluded that Nash's mixture (40% oil of citronella, 40% spirits of camphor, 20% oil of cedar) was the best. The repellent value of camphor and cedar oil in this mixture is not stated. Oil of citronella is extracted from *Andropogon nardus* (L.) and contains geraniol as its chief ingredient, others being citronellol, citronellal, borneol, and terpenes. The ingredients which repel mosquitoes have been found to be citronellol and the corresponding aldehyde, citronellal. Oil of citronella and citronella creams were the most popular repellents before 1938 (73). At that time, research on repellents dealt mainly with the study of essential oils and their constituents or with attempts to prolong their repellent action by nonvolatile oils or greases. Several current mixtures which are effective are listed in Table II. Formula No. 1 represents the well-known "6–2–2" repellent used by the military during World War II. The ingredients of this mixture were used individually as well. The indalone component of "6–2–2"

TABLE II
Mixtures of Repellents in Current Use

| Mixture | Per cent of components | Insects | Use[a] | Reference |
|---|---|---|---|---|
| 1. (6-2-2 or M-250) | | | | |
| Dimethyl phthalate | 60 | Mosquitoes, black flies, ticks, stable flies, sand flies | S&C | 2,35,90,98, 101,104 |
| Indalone | 20 | | | |
| 2-Ethyl-1,3-hexanediol | 20 | | | |
| 2. (M-2020) | | | | |
| Dimethyl phthalate | 40 | Mosquitoes, black flies, stable flies, sand flies, ticks | S | 2,34,35,92 |
| 2-Ethyl-1,3-hexanediol | 30 | | | |
| Dimethyl carbate | 30 | | | |
| 3. (M-2086) | | | | |
| Benzyl benzoate | 45 | Chiggers, ticks | C | 2,39,94 |
| di-*n*-Butyl phthalate | 45 | | | |
| Tween 80 | 10 | | | |
| 4. (M-1960) | | | | |
| *N*-*n*-Butylacetanilide | 30 | Ticks, chiggers, fleas, mosquitoes | C | 2,11,34,35, 39,91 |
| 2-*n*-Butyl-2-ethyl-1,3-propanediol | 30 | | | |
| Benzyl benzoate | 30 | | | |
| Tween 80 | 10 | | | |

[a] S denotes application to skin, C denotes impregnation into cloth.

repellent was replaced for military use by dimethyl carbate (see mixture No. 2) or *n*-propyl-*N*,*N*-diethyl succinamate.

The first repellent mixture (No. 3) developed for the treatment of clothing for protection against chiggers and which was used by the Armed Services, contained 45% each of benzyl benzoate and dibutyl phthalate and 10% of an emulsifier (94). Later a clothing treatment was developed for protection against several kinds of insects and contained 30% each of *N*-*n*-butylacetanilide, 2-butyl-2-ethyl-1,3-propanediol, and benzyl benzoate plus an emulsifier (mixture No. 4). In the Navy 448 mixture 2-cyclohexylcyclohexanol was substituted for 2,3,4,5-tetrahydro-2-naphthol when the latter was found to have toxicity (58).

There would seem to be little evidence for either synergism or antagonism between the components of a repellent mixture. Justification for the testing of mixtures of repellents developed for the purpose of repelling a broad spectrum of species may appear to be difficult since these mixtures represent a combination of particular repellents known to be most effective for the species in question. Smith *et al.* (92) found that "mixtures of several good repellents are effective against a wider range of species and on more individuals than any one repellent alone." This statement could be explained on the basis of merely additive effects of the mixed repellents rather than synergism. Because of chemical or physical incompatibility, however, certain combinations may be impractical. Some mixtures have been reported as effective repellents in which the repellent factor is unknown or is in natural combination with other compounds, such as the essential oils.

There exists a confusion over the concept of synergism and its definition. In order to avoid a lengthy description of contributing factors, we will accept the idea as given by Macht (69) and later by Sumerford (96) that synergism ["potentiation" of Goodman and Gilman (37)] describes a combined action of biologically active compounds with similar physiological action which is greater than that which is expected from the sum of their individual actions. Synergism thus should be differentiated from the activity of a mixture of two or more compounds whose joint action is a physical interaction not involving the biological systems, or whose joint action is the mere summation of similar or dissimilar effects on the organism.

In 1945, Pijoan, Jachowski and co-workers (15) described a change in repellent activity of some compounds, which was not attributable to dilution when the compounds were added to various nonrepellent solvents. A formula for deriving the "Synergic Index (SI)" was used to make this determination.

Wiesmann and Lotmar (109) do not claim" synergism" among the components of a mixture of *o*-chloro-*N*,*N*-diethylbenzamide, *N*,*N*-diethylbenzamide and isopropyl alcohol, but say that the observed enhancement of repellency over the separate ingredients is due to physical improvement, since the mixture was absorbed less by the skin. Roadhouse (81) states that 2-methyl-1-phenyl-1,2-propanediol is synergized in a 1:1 mixture with piperonal and in a 3.1 mixture with ethyl mandelate although the latter two compounds have negligible repellency alone. Howell and Goodhue (57) report that di-*n*-propyl-isocinchomeronate as well as MGK-264 (*n*-octyl-bicycloheptene-dicarboximide, a pyrethrum synergist) separately synergize 2,3,4,5-bis(2-butylene)-tetrahydrofurfural and 2,3,4,5-bis(2-butylene)-tetrahydrofurfuryl alcohol. Gilbert and Gouck (34) reported that 2-cyano-2-furanacrylate increased the effectiveness of dimethyl phthalate and two other mixtures.

The incorporation of repellent materials into lotions and creams reportedly has met with some success. In areas heavily infested with insects, such preparations conveniently could be used as part of the daily toilet. A solvent such as ethyl butyl cellosolve, being nonirritating, colorless, and with a faint odor reminiscent of witch hazel, was considered very useful in the inclusion of repellent materials into cosmetic creams since it resembles glycerol in its softening action on the skin (72). A vanishing cream containing dimethyl phthalate and pyrethrum was found to be effective as a repellent against a sand fly, *Phlebotomous papataci,* for a short time (85). Another improved cream containing dimethyl phthalate uses 15% acetostearin as a base (33).

There has been little effort directed toward the study of ingested chemicals for insect repellency. Shannon (87) reported that thiamin chloride in adequate dosage in humans diminished or might altogether eliminate the approach of mosquitoes toward the protected individual, in addition to alleviating itching from reactions following bites. Wilson *et al.* (110) in clearly defined experimental conditions found that with five human subjects thiamin chloride was not effective. However, in less-controlled studies, Eder (25) and Lunsford (68) found a protection against flea bites following treatment with thiamin chloride. Another orally administered repellent preparation, containing an extract of *Delphinium staphisagria,* is said to protect the majority of users against mosquito bites (64).

## IV. Chemical Structure and Repellency

One of the first studies to correlate chemical structure with repellency was made by Bunker and Hirschfelder (8) in gross field experiments with various genera of mosquitoes. Effectiveness was correlated with the presence of the oxygen atom, regardless of its position in the molecule. Roadhouse (81) reclassified the data of Morton *et al.* (75) and derived averages for various classes of compounds by adding the products of the indices of effectiveness and the number of the compounds of that rating in a particular chemical class, and dividing the sum by the total number of compounds in the class. The best class averages were found to have the highest fat and water solubility. Using these data and data from original experiments, he concluded that, from the standpoint of repellency, the most important atom seems to be oxygen, excluding compounds containing nitrogen atoms. Roadhouse also suggests "that the smell of a phenyl group is objectionable to insects," and that compounds (including 1,2- and 1,3-glycols) where the hydroxyl group is once removed from an unsaturation "enhance the power of repellency by increasing olfactory sensitivity to other objectionable groups." He does not define how a phenyl group can have a characteristic odor nor does he give supporting data for an increase in olfactory sensitivity.

From a series of field tests on stable flies, Moore (74) concluded that very slightly volatile, unsaturated cyclic esters were better than corresponding alcohols or hydrocarbons. In 1949, Travis *et al.* (102) classified 4300 organic compounds tested as repellents. The compounds effective for five hours or more were listed by classes in the following descending order of effectiveness: amides, imides > esters, lactones = alcohols (including phenols) > ethers, acetals > acids, anhydrides > halides = nitro compounds > amines = nitriles = other nitrogen compounds.

In a study of the structure and repellent action of new amides, Geigy and Utzinger (32) found that a diethylamide such as *N*,*N*-diethyl-alpha-aceto-beta-phenyl-propionamide is weakly repellent. Assuming that only the diethylamide moiety was effective, they made other related compounds and concluded that those amides with dimethyl groups on the same carbon atom combined with carbon to carbon double bonding repel well at first, but are not persistent, since they are unstable or are easily absorbed by the skin. Chlorine-substituted amides as a whole have good persistent effects, but many have a strong accessory effect on the arm of the user.

Barthel *et al.* (5) found that repellency decreased or was destroyed by ring substitution of mandelic acid esters with methoxy, methylenedioxy, or chlorine or by acylation of the alpha-hydroxy group. The maximum repellent effect (based on time of effectiveness) was observed with alkyl esters of six or fewer carbon atoms. The evaluation of esters rather than hydrocarbons affords a better chance of finding good repellents according to McCabe *et al.* (71). Ring-substituted diethylbenzamides were found to have high repellency except when substituted with hydroxy, alkoxy (higher than ethoxy), di- or tri-alkoxy, or di-halogen groups. Ortho-alkoxy substituted diethylbenzamides were better than the *p*-substituted isomers. However, the position of the ring substituent in alkyl-substituted diethylbenzamides appears to have no particular effect on repellent activity (71).

After careful consideration, it is easy to concur with Christophers (10) and Dethier (20) that while field tests are valuable to screen compounds in order to determine if they have the proper balance of attributes of a practical repellent, controlled laboratory studies are necessary to correlate repellency and structure with any degree of certainty. Not only is it necessary to control external and inherent variables, but also the experimental design must be rigid enough to withstand statistical analysis. A very good example of a well-designed experiment is that of Dethier and Yost (23). The problems inherent in studies of chemoreception are reviewed by Dethier and Chadwick (22) and Hodgson (51).

In studies designed to relate repellency of compounds to structure, significant data are frequently lost through the wrong choice of criteria of effectiveness or lack of adequate analysis. It is fallacious to express the concentration of the repellent in percentage when the stimulus is undoubtedly molecular. No conclusions were drawn by Leon *et al.* (63) about structure-activity of 12 esters of 1-hydroxycyclohexanecarboxylic acid, nor by Haller (49) from tests on a homologous series of phthalic acid esters, when considering molecular weight and repellent activity. Christophers (10) states that a boiling point of 280°C. is necessary if a substance is to be repellent for six hours and that any compound with a boiling point of 200°C. at 760 mm. may be disregarded. Roadhouse (81) found maximum repellent activity was invariably shown by compounds whose atmospheric boiling point was from 230° to 260°C. Some physical constants of a homologous series of compounds undergo approximately equal changes in passing from one member to the next (molecular weight, boiling point) while other properties, such as water solubility, surface activity, vapor pressure, partition coefficient, show the same logarithmic changes which characterize thresholds of toxicity and olfaction. This similarity in properties suggests an analysis of olfaction data with these thermodynamic activities as units of threshold measurement (18). Although the molecular weights (sometimes loosely referred to as "carbon-chain lengths") and boiling points of a homologous series should not be ignored, existing data suggest that other physical constants must be considered before final conclusions are made. Examples of this type of study may be cited in the work of Dethier and Yost (17,23). In studies of olfactory responses of the blow fly, *Phormia regina M.*, the median rejection threshold was the criterion of response. In terms of molar concentrations, homologous series of alcohols and aldehydes were rejected at logarithmically decreasing concentrations as the molecular weights increased. When thresholds were expressed as thermodynamic activities, most members of each series were approximately equally stimulating at rejection. The similarity between relative actions of the members of the two series suggests that the relation of equal stimulation at equal thermodynamic activities by homologous aliphatic compounds, at least for homologs of intermediate chain length, may be of rather general application in olfaction. The rejection response of blow fly receptors to pentanol vapors depends on stimulation of specialized receptors present on the antennae and palpi and hence cannot be attributed to activity of a common chemical sense. It was concluded further that a repellent in the gas phase undoubtedly acts as an olfactory stimulus. Different receptor fields function at different levels of sensitivity, with a relation between thresholds and the number of receptors functioning.

Although the chemoreceptors are very simple sense organs, their location and morphology have not been well established, except in a few cases. For more details see the reviews of Frings and Frings (28), Frings and O'Neal (29), Hodgson (51), and Dethier (16, 19). A definitive study was done on the cuticular structure of sense cells by Richards (80).

It is apparent from this review that there is a major need for standardized techniques for evaluating repellency. Most urgent is a method for measuring intrinsic repellency of compounds without the complicating variables inherent in host attractancy.

Development work is needed on the many effective compounds presently available in order to increase their time of repellency. This involves studies to prevent loss by skin absorption, evaporation, and sweat, and subsequent necessary formulation studies. It also involves a knowledge of host-insect relationships and, in the final analysis, of the mechanisms involved in repelling an insect from an attractive host. It is hoped that the next quarter century will be as fertile in the above respects as was the past quarter century in the empirical development of synthetic repellents.

## References

1. Altman, R., and Smith, C., Investigations of repellents for protection against mosquitoes in Alaska, 1953. *J. Econ. Entomol.*, *48*, 67–72 (1955).
2. Anonymous, Insecticides and repellents for the control of insects of medical importance to the Armed Forces. *U.S. Dept. Agr., Circ. No. 977* (1955).
3. Applewhite, K., and Cross, H., Further studies of repellents in Alaska. *J. Econ. Entomol.*, *44*, 19–22 (1951).
4. Applewhite, K., and Smith, C., Field tests with mosquitoes and sand fly repellents in Alaska. *J. Econ. Entomol.*, *43*, 353–7 (1950).
5. Barthel, W., Leon, F., and Hall, S., Insect repellents. I. Esters of mandelic and substituted mandelic acids. *J. Org. Chem.*, *19*, 485–9 (1954).
6. Brennan, J., Field tests with tick repellents. *U.S. Public Health Repts.*, *63*, 339–46 (1948).
7. Brody, A., A method for testing the value of chemical mixture as repellents of the Gulf Coast tick (*Amblyomma maculatum*). *U.S. Dept. Agr., Bur. Entomol. Plant Quarantine, ET-152* (1939).
8. Bunker, C., and Hirschfelder, A., Mosquito repellents. *Am. J. Trop. Med.*, *5*, 359–83 (1925).
9. Chapman, J., and Craig, R., An electrophysiological approach to the study of chemical sensory reception in certain insects. *Can. Entomologist, 85*, 182–9 (1953).
10. Christophers, S., Mosquito repellents. Being a report of the work of the Mosquito Repellent Inquiry, Cambridge 1943–5. *J. Hyg.*, *45*, 176–231 (1947).
11. Cole, M., and Lloyd, G., Field tests of selected repellents against the American dog tick. *J. Econ. Entomol. 44*, 1025–6 (1951).
12. Cross, H., and Snyder, F., Chigger control. *Soap Sanit. Chemicals, 25*, 135–43 (1949).
13. Davey, J., and O'Rourke, F., Observations of *Chrysops silacea* and *C. dimidiata* at Benin, Southern Nigeria. Part III. *Am. Trop. Med. and Parasitol.*, *45*, 101–9 (1951).
14. De Foliart, G., Comparison of several repellents against blackflies. *J. Econ. Entomol.*, *44*, 265–6 (1951).
15. Dethier, V., Chemical Insect Attractants and Repellents, Blakiston Co., New York, 1947.
16. Dethier, V., The relation between olfactory response and receptor population in the blowfly. *Biol. Bull.*, *102*, 11–17 (1952).
17. Dethier, V., Olfactory responses of blowflies to aliphatic aldehydes. *J. Gen Physiol.*, *37*, 743–51 (1954).
18. Dethier, V., The physiology of olfaction in insects. *Ann. N.Y. Acad. Sci.*, *58*, 139–57 (1954).
19. Dethier, V., The physiology and histology of the contact chemoreceptors of the blowfly. *Quart. Rev. Biology*, *30*, 348–71 (1955).
20. Dethier, V., Repellents. *Ann. Rev. of Entomol.*, *1*, 181–202 (1956).
21. Dethier, V., and Chadwick, L., Rejection thresholds of the blowfly for a series of aliphatic alcohols. *J. Gen. Physiol.*, *30*, 247–53 (1947).

22. Dethier, V., and Chadwick, L., Chemoreception in insects. *Physiol. Revs.*, *28*, 220–54 (1948).

23. Dethier, V., and Yost, M., Olfactory stimulation of blowflies by homologous alcohols. *J. Gen. Physiol.*, *35*, 823–39 (1952).

24. Draize, J., Alvarez, E., Whitesell, M., Woodard, G., and Hagan, E., Toxicological investigations of compounds proposed for use as insect repellents. A. Local and systemic effects following topical skin application. B. Acute oral toxicity. C. Pathological examination. *J. Pharmacol. Exptl. Therap.*, *93*, 26–39 (1948).

25. Eder, H., Flea bites: prevention and treatment with thiamin chloride. *Arch. Pediatrics*, *62*, 300 (1945).

26. Eddy, G., and McGregor, W., Use of white mice for testing materials as repellents and toxicants for stable flies. *J. Econ. Entomol.*, *42*, 461–3 (1949).

27. Findlay, G., Jardwicke, J., and Phelps, A., Tsetse fly repellents. *Trans. Roy. Soc. Trop. Med. Hyg.*, *40*, 341–4 (1946).

28. Frings, H., and Frings, M., The loci of contact chemoreceptors in insects. *Am. Midland Naturalist*, *41*, 602–58 (1949).

29. Frings, H., and O'Neal, B., The loci and thresholds of contact chemoreceptors in females of the horsefly, *Tabanus sulcifrons* Macq. *J. Exptl. Zool.*, *103*, 61–79 (1946).

30. Fryer, H., Atkeson, F., and Smith, R., Comparison of methods for testing repellent-type fly sprays. *J. Econ. Entomol.*, *41*, 80–8 (1948).

31. Fryer, H., Shaw, A., Atkeson, F., Smith, R., and Borgmann, A., Techniques for conducting fly-repellency tests on cattle. *J. Econ. Entomol.*, *36*, 33–44 (1943).

32. Geigy, R., and Utzinger, G., Structure and repellent action of new amides, and the employed test procedures. *Acta Tropica*, *10*, 349–60 (1953).

33. Gerding, P., Sperandio, G., and Christian, J., The retention of an insect repellent in emulsified bases at the site of application. *J. Am. Pharm. Assoc.*, *44*, 574–7 (1955).

34. Gilbert, I., and Gouck, H., Evaluation of repellents against mosquitoes in Panama. *Florida Entomologist*, *38*, 153–63 (1955).

35. Gilbert, I., Gouck, H., and Smith, C., New mosquito repellents. *J. Econ. Entomol.*, *48*, 741–3 (1955).

36. Goodhue, L., and Stansbury, R., Some new fly repellents from laboratory screening tests. *J. Econ. Entomol.*, *46*, 982–5 (1953).

37. Goodman, L., and Gilman, A., The Pharmacological Basis of Therapeutics, Macmillan, New York, N.Y., 1946.

38. Goodwin, W., Sloan, M., and Schwardt, H., Repellency test for horse flies and horn flies in N.Y. State. *J. Econ. Entomol.*, *45*, 121–2 (1952).

39. Gouck, H., and Gilbert, I., Field tests with new tick repellents in 1954. *J. Econ. Entomol.*, *48*, 499–500 (1955).

40. Granett, P., Mosquito repellents. 1. Test procedure and method of evaluating test data. *J. Econ. Entomol.*, *33*, 563–5 (1940).

41. Granett, P., The development of a practical mosquito repellent. *Proc. New Jersey Mosquito Exterm. Assoc.*, *27*, 36–43 (1940).

42. Granett, P., "Paired product testing" for the evaluation of mosquito repellents. *Proc. New Jersey Mosquito Externi. Assoc.*, *31*, 173–8 (1944).
43. Granett, P., and French, C., Field tests of clothing treated to repel American dog ticks. *J. Econ. Entomol.*, *43*, 41–4 (1950).
44. Granett, P., and French, C., Dibutyl adipate as a tick repellent. *J. Econ. Entomol.*, *44*, 93–7 (1951).
45. Granett, P., and Haynes, H., Insect-repellent properties of 2-ethyl-1,3-hexanediol. *J. Econ. Entomol.*, *38*, 671–5 (1945).
46. Granett, P., Haynes, H., Connola, D., Bowery, T., and Barber, G., Two butoxypolypropylene glycol compounds as fly repellents for livestock. *J. Econ. Entomol.*, *42*, 281–6 (1949).
47. Granett, P., Haynes, H., and Helm, R., Butoxypolypropylene glycol as a fly repellent for dairy cattle. *J. Econ. Entomol.*, *44*, 97–102 (1951).
48. Granett, P., and Sacktor, B., Testing tick repellents and observations of phototropic effects. *J. Econ. Entomol.*, *40*, 259–63 (1947).
49. Haller, H., Wartime development of insecticides. *Ind. Eng. Chem.*, *39*, 467–73 (1947).
50. Healy, M., A method for comparing fly repellent sprays. *Biometrics*, *9*, 290–4 (1953).
51. Hodgson, E., Problems in invertebrate chemoreception. *Quart. Rev. Biology*, *30*, 311–47 (1955).
52. Hodgson, E., Lettvin, Y., and Roeder, K., Physiology of a primary chemoreceptor unit. *Science*, *122*, 417–18 (1955).
53. Horn, D., Nephrosis probably due to excessive use of Sta-way insect repellent. *J. Am. Med. Assoc.*, *128*, 513 (1945).
54. Howard, L., Remedies and preventives against mosquitoes. *Farmer's Bull. No. 444, U.S. Dept. Agr.*, 1923.
55. Howell, D., and Fenton, F., Repellency of a pyrethrum-thiocyanate oil spray to flies attacking cattle. *J. Econ. Entomol.*, *37*, 677–80 (1944).
56. Howell, D., and Goodhue, L., Laboratory and field tests on fly repellents. *Chem. Specialties Mfrs. Assoc. Proc.*, *1955*, 111–14 (1955).
57. Howell, D., and Goodhue, L., Fly repellents. *Soap Sanit. Chemicals*, *55*, 181, 185–9, 221 (1955).
58. Jachowski, L., and Pijoan, M., Two new effective insect repellents, NMRI-201 and NMRI-448. *Science*, *104*, 266–9 (1946).
59. Kasman, S., Roadhouse, L., and Wright, G., Testing of insect repellents. *Mosquito News*, *13*, 116–23 (1953).
60. King, W., Some results of recent work on the newer insecticides. *Am. J. Trop. Med.*, *28*, 487–97 (1948).
61. King, W., Chemicals evaluated as insecticides and repellents at Orlando, Florida. *Agr. Handbook 69*, 1954.
62. Kuhn, H., Developments in the German chemical industry. Data collected by Industrial Intelligence Staff, Chemical Warfare Service. *Chem. Eng. News*, *23*, 1516–22 (1945).
63. Leon, J., Barthel, W., and Hall, S., Insect repellents. II. Esters of 1-hydroxycyclohexanecarboxylic acid. *J. Org. Chem.*, *19*, 490–2 (1954).

64. Lesser, M., Modern insect repellents. *Drug & Cosmetic Ind.*, *75*, 762–3, 862–7 (1954).

65. Lindsay, I., An olfactometer for examining the effect of vapors on adult mosquitoes. *Suffield Tech. Paper, Can. D.R.B. No. 76* (1955).

66. Linduska, J., Cockran, J., and Morton, F., Flea repellents for use on clothing. *J. Econ. Entomol.*, *39*, 767–9 (1946).

67. Lord, K., and Johnson, C., Production of dermatitis by pyrethrum and attempts to produce a nonirritant extract. *Brit. J. Dermatol. Syphilis*, *59*, 367–75 (1947).

68. Lunsford, C., Newer insecticides and scabicides. *Calif. Med.*, *72*, 350–1 (1950).

69. Macht, D., Pharmacological synergism of stereoisomers. *Proc. Nat. Acad. Sci.*, *15*, 63–70 (1929).

70. Mailen, T., and Fenton, F., Repellency in cattle sprays. *Soap Sanit. Chemicals*, *17*, 133, 135 (1941).

71. McCabe, E., Barthel, W., Gertler, S., and Hall, S., Insect repellents. III. N,N-Diethylamides. *J. Org. Chem.*, *19*, 493–8 (1954).

72. McClure, H., Newer aliphatic chemicals. *Chem. Eng. News*, *22*, 416–21 (1944).

73. McCulloch, R., and Waterhouse, D., Laboratory and field tests of mosquito repellents. *Australia Council Sci. Ind. Research, Bull. No. 213*, 9–28 (1947).

74. Moore, W., Esters as repellents. *J. N. Y. Entomol. Soc.*, *42*, 185–92 (1934).

75. Morton, F., Travis, B., and Linduska, J., Part V. Evaluation of materials as mosquito repellents. *In* Results of screening tests with materials evaluated as insecticides, miticides and repellents at the Orlando laboratory, April 1942 to April 1947, pp. 10–11, 22–235. *U.S. Dept. Agr., Bur. Entomol. Plant Quarantine, E-733* (1947).

76. Patton, T., and Favorite, F., Human toxicological aspects of the insecticide and insect repellent program of the Department of Army. *Proc. New Jersey Mosquito Exterm. Assoc.*, *38*, 50–4 (1951).

77. Pearson, A., An improved method for the determination of cattle fly spray repellence. *J. Econ. Entomol.*, *28*, 160–1 (1935).

78. Peterson, D., A report on the assessment of biting-fly repellents in Canada since 1952. *Can. Dept. Agr. Publ.-23* (1956).

79. Pijoan, M., New insect repellents. *Soap Sanit. Chemicals*, *23*, 124–7, 173, 175 (1947).

80. Richards, A., Studies on arthropod cuticle. VIII. The antennal cuticle of honeybees with particular reference to the sense plates. *Biol. Bull.*, *103*, 201–25 (1952).

81. Roadhouse, L., Laboratory studies on insect repellency. *Can. J. Zool.*, *31*, 535–46 (1953).

82. Roth, A., Mote, D., and Linquist, D., Tests of repellents against tabanids. *U.S. Dept. Agr., Agr. Research Service, Entomol. Res. Bur. ARS-33-2* (1954).

83. Roy, D., and Ghosh, S., Further work on the comparative efficacy of different culicifuges under laboratory conditions. *Parasitol.*, *34*, 291–4 (1942).

84. Roys, C., Olfactory nerve potentials a direct measure of chemoreception in insects. *Ann. N.Y. Acad. Sci.*, *58*, 250–5 (1954).
85. Sabin, A., Philip, C., and Paul, J., Phlebotomus (Pappataci or Sandfly) fever. *J. Am. Med. Assoc.*, *125*, 693–9 (1944).
86. Sarkaria, D., and Brown, A., Responses of the female Aedes mosquito. II. Action of liquid repellent compounds. *Bull. Entomol. Research*, *42*, 115–22 (1951).
87. Shannon, W., Thiamine chloride—an aid in the solution of the mosquito problem. *Minnesota Med.*, *26*, 799–802 (1943).
88. Smith, C., New materials for use as insect repellents. *Chem. Specialties Mfrs. Assoc. Proc.*, Dec. *1950*, 80–1.
89. Smith, C., and Burnett, D., Jr., Effectiveness of repellents applied to clothing for protection against salt-marsh mosquitoes. *J. Econ. Entomol.*, *42*, 439–44 (1949).
90. Smith, C., and Gouck, H., Tick repellents. *J. Econ. Entomol.*, *39*, 374–8 (1946).
91. Smith, C., Cole, M., Gilbert, I., and Gouck, H., Field tests with tick repellents—1949, 1950 and 1952. *J. Econ. Entomol.*, *47*, 13–19 (1954).
92. Smith, C., Cole, M., Lloyd, G., and Selhime, A., Mosquito-repellent mixtures. *J. Econ. Entomol.*, *45*, 805–9 (1952).
93. Snyder, F., and Morton, F., Chigger repellents. *Soap Sanit. Chemicals*, *22*, 11, 133, 135 (1946).
94. Snyder, F., and Morton, F., Benzyl benzoate-dibutyl phthalate mixture for impregnation of clothing. *J. Econ. Entomol.*, *40*, 586–7 (1947).
95. Starnes, E., and Granett, P., A laboratory method for testing repellents against biting flies. *J. Econ. Entomol.*, *46*, 420–3 (1953).
96. Sumerford, W., Review of synergism among halogen-containing insecticides and halogen-containing synergists. *Agr. and Food Chem.*, *2*, 310–27 (1954).
97. Travis, B., Mosquito and other biting-insect problems in Alaska. *J. Econ. Entomol.*, *42*, 451–7 (1949).
98. Travis, B., and Morton, F., Treatment of clothing for protection against mosquitoes. *Proc. New Jersey Mosquito Exterm. Assoc.*, *33*, 65–9 (1946).
99. Travis, B., and Morton, F., Insect repellents and nets for use against sand flies. *Proc. New Jersey Mosquito Exterm. Assoc.*, *37*, 154–6 (1950).
100. Travis, B., and Morton, F., Use of insect repellents and toxicants. *Bur. Entomol. Plant Quarantine E-698*, 2nd rev., 1953.
101. Travis, B., Morton, F., and Cochran, J., Insect repellent used as skin treatment by the armed forces. *J. Econ. Entomol.*, *39*, 627–30 (1946).
102. Travis, B., Morton, F., Jones, H., and Robinson, J., The more effective mosquito repellents tested at the Orlando, Florida, Laboratory. 1942–1947. *J. Econ. Entomol.*, *42*, 686–94 (1949).
103. Travis, B., and Smith, A., Tests with repellents against the stable fly. *J. Am. Vet. Med. Assoc.*, *119*, 214–16 (1951).
104. Travis, B., Smith, A., and Madden, A., Jr. Effectiveness of insect repellents against black flies. *J. Econ. Entomol.*, *44*, 813–14 (1951).

105. Travis, B., and Smith, C., Materials for protection against biting insects. *J. Forestry*, *48*, 329–30 (1950).

106. Twinn, C., The newer insecticides, repellents, and rodenticides of value in the field of public health. *Sci. Agr.*, *27*, 97–104 (1947).

107. Wadley, F., Incomplete-block design adapted to paired tests of mosquito repellents. *Biometrics Bull.*, *2*, 30–1 (1946).

108. Wasicky, R., Unti, O., and daSilva Ramos, A., Repellents for Culex species. *Anais fac. farm. e odontol. univ. São Paulo*, *7*, 297–305 (1949).

109. Wiesmann, R., and Lotmar, R., Field of action of the new repellent. *Acta Tropica*, *6*, 292–349 (1949).

110. Wilson, C., Mathieson, D., and Jachowski, L., Ingested thiamine chloride as a mosquito repellent. *Science*, *100*, 147 (1944).

# THE STATUS OF SYSTEMIC INSECTICIDES IN PEST CONTROL PRACTICES

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## I. Introduction

This review summarizes information on the status of systemic insecticides in pest control of phytophagous pests only: shortage of space does not allow a discussion of the medication of animals by systemic insecticides to eradicate parasitic or blood-sucking insects. Since the author's last review (177) a bibliography compiled by Giang (74) was published in 1953 and a German review was published by Spindler (208) in 1955. A good deal of the literature has been collected and reviewed in a chapter of Metcalf's *Organic Insecticides* (148) and in articles by Greenslade (80), Ivy (112), Jeppson (118), Reynolds (174), Unterstenhöfer (222), von Rümker (187–189), and Metcalf (150); further in Spencer and O'Brien's review, "The Chemistry and Mode of Action of Organophosphorus Insecticides" (207) and in Bennett's (9) review of the behavior of systemic insecticides applied to plants.

Systemic or endotherapeutic insecticides have been defined as compounds which are absorbed by the plant and translocated in quantities sufficient to make the site of translocation insecticidal (9), whereby these compounds are stored for a limited time in the plant on the site of translocation (221).

The last two criteria distinguish systemic insecticides from penetrating insecticides which possess translaminar action but are not translocated *and* stored in insecticidal quantities; the latter are referred to in German literature as insecticides with "Tiefenwirkung" (221). Penetrating insecticides are sometimes translocated but the quantities translocated are so small a proportion of those applied that these insecticides are not classified in the systemic group. "Tiefenwirkung" has been established for BHC (9,61,82,83,131, 132,133,201), parathion (20,65,103,135,136,172,193,201,239), malation, Hercules 528 (190), Guthion® or *O,O*-dimethyl *S*-3-(4-ketobenzotriazine) methyl phosphorodithioate, nicotine (41), cyanamide (66,128), quassia (3), rotenone (69), and diazinon (70,71,72,126). A compound with an intermediate position to true systemics is paraoxon (42,45,112).

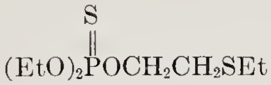
According to Metcalf (150) the controlling properties for systemic action appear to be: (a) sufficient water solubility to enable the compound to move in the plant sap; (b) ability to penetrate into the plant through roots, leaves, and stem; (c) sufficient stability in the plant environment to enable the systemic compound to exert the required degree of residual insecticidal action. To this must be added, in order to make the systemic compound acceptable for public health reasons, a further property, viz., that this compound is subject to decomposition in the plant tissue to nontoxic products, within a reasonable time, i.e., 3–5 weeks.

A method of classification of the systemic insecticide has been proposed which is based on the fate of the systemic insecticide in the plant (177) because the form in which insecticidal compounds are taken up by the phytophagous insect is of considerable importance. The classification is threefold:

1. Stable systemic insecticides which are not metabolized by the plant.
2. Endolytic systemic insecticides in which the toxic compound is present to 98% in its original form when taken up by the insect until it is finally decomposed by the plant.
3. Endometatoxic systemic insecticides which are transformed in the plant partially or wholly into other toxic substances which also

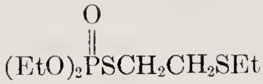
act as insecticides when they are taken up by the pest, before they are decomposed by the plant.

The discovery of endometatoxicity was made by Hartley (88,89) and Heath (97) for demeton; the oxidation products of the demeton isomers, *O,O*-diethyl *O*-ethyl-2-mercaptoethyl phosphorothionate (I), and *O,O*-diethyl *S*-ethyl-2-mercaptoethyl phosphorothiolate (II) were first described by Heath *et al.* (97,130) and were found to be a thousand times more cholinergic than their parent substance. Metcalf and his collaborators confirmed this and further explored the products of enzymatic decomposition of the demeton isomers in a number of classical papers (68,137,138,139,152,153,154). Fukuto *et al.* (68a, 68b) found seven oxidation products of thiono and thiol isomers of demeton, of which the most toxic metabolites were the *O,O*-diethyl *O*-ethyl-2-sulfinylethyl phosphorothionate (III) (thionophosphate sulfoxide), *O,O*-diethyl *S*-ethyl-2-sulfinylethyl phosphorothiolate (IV) (thiolphosphate sulfoxide) and the thiolphosphate sulfone, *O,O*-diethyl *S*-ethyl-2-sulfonylethyl phosphorothiolate (V). Methyl demeton is metabolized in an analogous way (130,157) and 3911 (Thimet®)



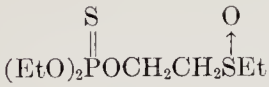
$$(\mathrm{EtO})_2\overset{\mathrm{S}}{\overset{\|}{\mathrm{P}}}\mathrm{OCH_2CH_2SEt}$$

(I)



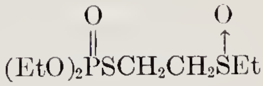
$$(\mathrm{EtO})_2\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}\mathrm{SCH_2CH_2SEt}$$

(II)



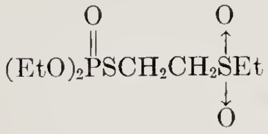
$$(\mathrm{EtO})_2\overset{\mathrm{S}}{\overset{\|}{\mathrm{P}}}\mathrm{OCH_2CH_2}\overset{\mathrm{O}}{\overset{\uparrow}{\mathrm{S}}}\mathrm{Et}$$

(III)



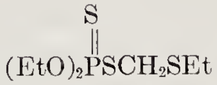
$$(\mathrm{EtO})_2\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}\mathrm{SCH_2CH_2}\overset{\mathrm{O}}{\overset{\uparrow}{\mathrm{S}}}\mathrm{Et}$$

(IV)



$$(\mathrm{EtO})_2\overset{\mathrm{O}}{\overset{\|}{\mathrm{P}}}\mathrm{SCH_2CH_2}\underset{\mathrm{O}}{\underset{\downarrow}{\overset{\mathrm{O}}{\overset{\uparrow}{\mathrm{S}}}}}\mathrm{Et}$$

(V)



$$(\mathrm{EtO})_2\overset{\mathrm{S}}{\overset{\|}{\mathrm{P}}}\mathrm{SCH_2SEt}$$

(VI)

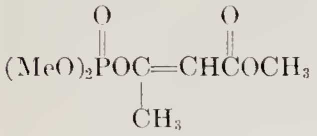
or *O,O*-diethyl *S*-ethylmercaptomethyl phosphorodithioate (VI) is oxidized to the dithiophosphate sulfoxide and dithiophosphate sulfone and finally to monothiophosphate sulfone. Again the oxidative metabolites are more potent cholinesterase inhibitors than Thimet® and are finally hydrolyzed to diethyl phosphoric acid and diethyl thiophorphoric acid which both show no appreciable toxicity (5).

When a susceptible pest ingests plant juices containing certain systemic insecticides a complex with the anticholinesterase enzyme is directly formed. As this phosphate-enzyme complex is only very slowly hydrolyzed, the enzyme is blocked and thus is unavailable for acetyl choline decomposition. "Directly toxic" systemic insecticides are Phosdrin® or *O,O*-dimethyl *O*-2-carbomethoxy-1-methyl vinyl phosphate (VII) the oxidative metabolites of both demeton isomers, of methyl demeton, dithiodemeton (VIII) and Thimet®, which are produced in the plant, and monochlorinated schradan; this was synthesized by Spencer and O'Brien who demonstrated a 100,000-fold enhancement of the anticholinesterase activity of schradan by introducing an electrophilic group (206).

By contrast there are systemic insecticides which derive their toxic action from an enzymatic metabolization by the insect into a cholinesterase inhibitor which, in the case of the susceptible species, kills the insect; I propose to call these *zoometatoxic* systemic insecticides. The best studied example is schradan (IX). In this case, the metabolic oxidation in the plant is so slow that an oxidation product is only present in plant juices which an insect takes up in unimportant quantities but the oxidation takes place in the insect (27,49,58). It therefore seems logical to class schradan among the endolytic systemics although very small quantities of its oxidation product are produced in the plant, because their unimportance from the insecticidal point of view has clearly been demonstrated by De Pietri-Tonelli *et al.* (49). To provide for such a relatively unimportant oxidation product present in traces only, an endolytic systemic was defined in 1952 as having 98% of the insecticidal compounds present in their original form (177). There is still some controversy on the oxidation of schradan to the octamethyl pyrophosphoramide *N*-oxide of schradan (X) (26,27,91,95,124,220) which is a million times more cholinergic than schradan. As to the constitution of the metabolite, Hartley (87), Ripper, Greenslade, and Hartley (182), and Heath, Lane, and Llewellyn (95) suggested a coordination of an oxygen into the tertiary nitrogen of the dimethylamido group, and Casida *et al.* (27) have produced evidence that the monophosphoramidoxide of schradan is the most cholinergic metabolite.

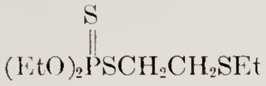
O'Brien and Spencer (164b) suggested that this active anticholinesterase might be the amidoxide (X) or the methylol derivative (XI). Tsuyuki *et al.* (220) concluded that the cholinergic metabolite is the amidoxide (X), but Heath *et al.* (96) showed that the partition coefficient of the active component fits the methylol (XI) structure better.

Spencer and O'Brien (207) suggested therefore that, in the metabolic as well as the chemical oxidation of schradan, the amidoxide is a transitory intermediate and concur with Heath *et al.* (96) that the active anticholinesterase is the methylol derivative.



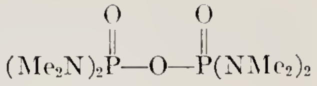
$(MeO)_2\overset{O}{\overset{\|}{P}}O\underset{CH_3}{\underset{|}{C}}{=}CH\overset{O}{\overset{\|}{C}}OCH_3$

(VII)



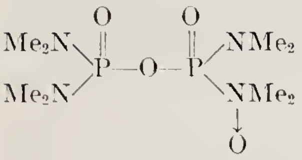
$(EtO)_2\overset{S}{\overset{\|}{P}}SCH_2CH_2SEt$

(VIII)

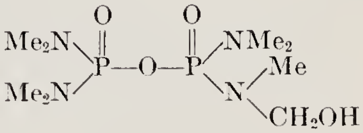


$(Me_2N)_2\overset{O}{\overset{\|}{P}}{-}O{-}\overset{O}{\overset{\|}{P}}(NMe_2)_2$

(IX)



(X)



(XI)

For pest control practices the distinction between "directly toxic" and zoometatoxic systemics is important because "directly toxic" substances, other things being equal, kill faster as Casida has shown for the oxidative metabolite of schradan (27).

Another factor which has great bearing on the performance of a systemic insecticide is the persistence of the systemic toxicity in the plant; this depends on maintaining in the plant tissue where the pests feed a differential between the amount of the toxic compound or its precursor translocated or stored on the one hand, and the decomposition, evaporation, and excretion (167,219) of the toxic compound on the other in excess of the lethal doses. Persistence is therefore related to the dosage applied (6,134). The shortest residual period was found by Casida with the substituted vinyl phosphates when, after the initial loss by evaporation, the enzymatic detoxification yielded a half-life in young greenhouse pea plants of 20 hours for *cis*-OS 2046 and 48 hours for the *trans* isomer (24,28). The longest residual effect during the growing season was 32–35 days obtained with schradan and diethyl ethylselenoethylthiophosphate.

Progress has been made in the acceptance of the use of systemic organophosphorus insecticides of high toxicity to mammals because it was possible to show for the cholinesterase inhibiting systemic insecticides that thorough precautionary measures are adequate to avoid the hazards of application and are fully practicable (23,60,92, 93,224). Intensive research work in the last seven years has revealed more fully the mode of action of these compounds (7,25,55, 144,165,207,237,238) than that of other pesticides used and it has been demonstrated that the routes of metabolism of systemics such as schradan, demeton and similar compounds and their toxic metabolites are the same in plants, insects, and mammals so that toxic residues in edible produce present no peculiar hazard as a result of biochemical activity of the plant (March *et al.* (139)); the metabolization in the plant leads to very low residues at harvest time, provided the application has been made sufficiently early or, if closer to harvest, at much reduced concentration (Davich and Apple (38), Metcalf *et al.* (153)). Adequate microanalytical methods are now available for many systemic insecticides (8,57,94,105,123,138,166) to determine very small residues in agricultural produce.

Suitable tolerance limits for systemic insecticides in agricultural produce for human consumption have been reviewed in the various user countries. For schradan a level of 3 p.p.m. was suggested in Britain for supplementary items of diet, i.e., popular vegetables and fruit (39,177) and a level of 1 p.p.m. has been recommended as safe by the British Ministry of Agriculture for brussels sprouts, sugar beet, field beans, cauliflower, cabbage, cucumber, strawberries, apples, and hops. For dimefox, a tolerance of 0.1 p.p.m. has been accepted as safe by the Food and Drug Administration in the U. S. A. for imported cocoa beans, and in Britain dimefox has been approved by the Ministry of Agriculture for use on sugar beets, hops, and mangolds. Neither of these compounds has been issued a tolerance in the United States under the Miller Bill. Systox® has, however, been granted a tolerance under this law of 0.75 p.p.m. on apples, broccoli, brussels sprouts, cabbage, cauliflower, musk melons, oranges, pears, potatoes, strawberries, and walnuts; and a tolerance of 0.3 p.p.m. was granted for beans. These values are based on a determination by the cholinesterase enzyme assay, using technical Systox® as a standard, which is a weak inhibitor. As Metcalf *et al.* (153) have pointed out, demeton is rapidly converted into more cholinergic metabolites so that residues determined by this technique and recalculated as demeton are 5–10 times too great. Experience over 7 years has shown that residues of systemic insecticides at harvest time are minimal if the recommended time interval between application and harvest time is observed (177). This is due to several factors: (a) high insecticidal effectiveness requires only low rates of application; (b) prolonged tenacity of the insecticidal action resulting in fewer treatments; (c) a poor degree of translocation into: fruits, pods, hop cones, beans, seeds, and roots found after treatment (38); and last but not least, (d) the rapid penetration into the plant where the systemic insecticides are exposed to decomposition by enzymatic action and aqueous hydrolysis. Metcalf has recently compiled a table which is here reproduced to illustrate this point (150). (Table I.)

TABLE I

Residues of Systemic Insecticides in Produce at Harvest
(Heath *et al.* (95), Metcalf *et al.* (153), Casida *et al.* (28))

| Crop | Dosage | Days after application | Residue, p.p.m. |
|---|---|---|---|
| | Schradan | | |
| Strawberries | 2 lb./acre | 30 | 1.17–2.36 |
| Sugar beet | 8 lb./acre | 40 | 0.0–0.7 |
| Hops | 2.5 lb./acre | 42 | 1.5 |
| Brussels sprouts | 7.5 lb./acre | 34 | 0.51–0.75 |
| | Dimefox | | |
| Cocoa beans | 250 p.p.m. of tree wt. | 21 | 0.01–0.03 |
| Coffee | 9 g. per bush | 270 | 0.02 |
| Potatoes | 12 lb./acre, 3 times | 42 | 0.08 |
| | Systox® | | |
| Orange juice | 4 oz./100 gal. | 28 | 0.013 |
| Orange peel | 4 oz./100 gal. | 28 | 0.24 |
| Apples | 2 oz./100 gal. | 30 | 0.0068 |
| Pears | 2 oz./100 gal. | 30 | 0.056 |
| Walnut meats | 4 oz./100 gal. | 30 | 0.0095 |
| Potatoes | 4 oz./100 gal. | 33 | 0.052 |
| Sugar beet | 4 oz./100 gal. | 30 | 0.130 |
| | Phosdrin® | | |
| Cabbage | 0.25 lb./acre | 4 | <0.04 |
| Snap beans | 0.25 lb./acre | 4 | <0.04 |

By contrast, fluoroacetate has not been accepted in Europe and the U. S. A. for crop protection because of its high toxicity to mammals and the lack of an early diagnosis of accidental intake before toxic symptoms arise, but sodium monofluoroacetate is marketed in Japan commercially and the intention to market it for nonfood crops has been notified in Great Britain. Selenium compounds have also not been accepted although some of the newer compounds synthesized by Schrader (194,195) are among the most effective systemic insecticides so far made (145). The Food and Drug Administration stipulates a nil residue in the harvested produce; as many soils contain small quantities of selenium, an opposition to the use of such compounds because of the stability of selenium compounds in the soil is hardly logical if very small quantities per acre are used, so that a contamination of the soil to the extent of rendering it unsuitable for following food crops is not effected. But in view of the high price of the selenium and its very high toxicity to mammals, considerable offsetting advantages over sulfur in organic phosphorus compounds would be required to justify its use.

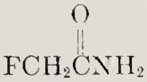
## II. Application Methods and Their Plant Physiological Basis

### A. FOLIAR SPRAYS

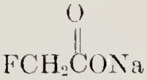
The ability of the leaf to absorb systemics depends (i) on the retention of the spray, (ii) on evaporation, (iii) the speed at which the spray droplet dries, and (iv) anatomical structures of the leaf and in particular its cuticle.

David has established a rate of absorption for the water-soluble schradan (39) and dimefox (40) and Tietz submitted data indicating a quicker absorption of the nearly water-insoluble demeton (219). This is not in agreement with the study of the behavior of demeton isomers in plants by Metcalf *et al.* who found the maximum concentration of the thiono isomer of demeton or its metabolites in lemon leaves was only about one-fifth that obtained with schradan when applied in equal concentration under similar conditions. When leaf applications were made with the thiol isomer, the absorption and translocation was rapid, 5–10 times that of the thiono isomer, and the total concentration in the leaves was of the same magnitude as that obtained with schradan (152), while the sulfoxide of the thiol isomer is even less readily absorbed than the thionoisomer (154). The conversion of both isomers was so rapid that in 4 days 97–100% of the demeton homologues was metabolized.

Following leaf applications with the thiol isomer of demeton, Thomas *et al.* (217) found three processes occurred concurrently, namely evaporation, breakdown into toxic nonvolatile compounds, and absorption so that the unchanged thiol isomer was removed from the surface within a few hours. By contrast, $P^{32}$ tagged schradan was partially absorbed by leaves of apples, broad and runner beans, coleus and chrysanthemums, some evaporated, and the rest remained for a considerable time on the leaf surface exposed to removal by aqueous leaching. Monofluoroacetamide (XII) penetrates leaves rapidly according to Takeuchi, Horiuchi, and Kuwada (213) and is in this respect better than sodium monofluoroacetate (XIII), while $\beta$-fluoroethyl fluoroacetate (XIV) did not penetrate leaves well.


$\mathrm{FCH_2}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}\mathrm{NH_2}$

(XII)


$\mathrm{FCH_2}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}\mathrm{ONa}$

(XIII)

$\mathrm{FCH_2}\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}\mathrm{OCH_2CH_2F}$

(XIV)

Stomatal ridges, the basal cells of hairs, and anticlines of the epidermis cells are thought to be sites of absorption (218,219).

Contrary to Zattler's contention that systemics are taken up by the stomata (241), Tietz (219) and Bennett and Thomas (12) confirm Heath and Llewellyn's evidence for a cuticular entry of organophosphorus compounds by showing that the number of stomata is not correlated with the uptake of leaves where the number of stomata varies on upper and lower surface. Lower surfaces of leaves of some plants show a more retentive surface and therefore give higher uptake values; in such cases lower surface sprays are cheaper to use. Bennett and Thomas's early finding of the importance of light for absorption (11) and Heath and Llewellyn's conclusion that absorption was sensitive to infrared and visible radiation has been amplified by Bennett and Thomas (12). Absorption in beans was greatly increased by raising the temperature even when the plants were screened from direct radiation, this being an effect on the rate and not the capacity of absorption; thus while at higher temperatures daylight does not increase the rate of absorption, it does so at lower temperatures, and at both ranges of temperature, light seemed to increase the absorptive capacity. Bennett and Thomas' experiments show that light increased absorption of chrysanthemums and coleus at all the temperatures prevailing.

Temperature and illumination have then important effects on absorption; as Davson and Danielli (48) have suggested that molecules must possess a critical energy of translocation of the barriers of the plasma membrane, it may be assumed that in conditions in which the critical energy required is larger, a rise in temperature may cause a larger number of molecules to permeate the membrane. Bennett and Thomas (12) therefore pointed out that at optimum conditions, cell membranes are in their most permeable state, but that there will be variation from species to species. When the conditions for a plant species are sub-optimal, the relative effectiveness of any factor is assessed by its ability to make the conditions approach optimum, and thus in some cases light, and in others temperature increases absorption of systemic insecticides. Young leaves were more absorptive to systemic insecticides than older leaves (12,199).

In the leaf the systemic insecticide applied by foliar sprays is not spread over large distances as a result of diffusion. The leaf veins inhibit the spray as the connecting bundle parenchyma restricts the movement of the systemic insecticides (199,217,219). When single leaves are treated, the systemic insecticides schradan and demeton move to untreated parts of the plant; the translocation toward the apex and upward is more intensive than in the basal direction (199, 216,219).

Metcalf *et al.* (151,152), Thomas and Bennett (216) and Thomas, Bennett and Lloyd-Jones (217) confirmed earlier findings (10,181,182, 232) that the translocation of schradan and demeton isomers is toward the younger leaves and the peripheral growing areas of the upper leaves. The translocated quantities of systemic insecticides are only insecticidal when the greater part of the plant is treated with a solution at the recommended concentrations. From this it follows that foliar applications must procure a reasonably good cover (117). The translocation of the systemic insecticide can be exploited by the use of low volume sprays but a number of droplets must be applied to each leaf. This enables sizable economies in water transportation but the extent of the translocation should not be overestimated and, on other than field crops, the application rate of the low volume sprays should not be too small. Practical experience and plant physiological research are in good agreement on this point. The extent to which very low volume can be used depends on the $LD_{50}$ of the systemic insecticide and the location of the pest on the plant. On cotton, low volume application by airplane down to one gallon using schradan and methyl demeton has proved successful, but application of less than three-quarters of a gallon to 4-foot tall cotton using forward-directed fan nozzles at 45 lb. pressure and flying at 60 m.p.h. no longer gave a complete kill of *Aphis gossypii* (Glover) (183).

The translocation of systemic insecticides applied by foliar treatment occurs primarily in the phloem together with the products of assimilation although certain quantities diffuse in the xylem and are translocated particularly upward, as has been shown by Tietz for demeton (219) and by Thomas and Bennett (216) and Wedding (231) for schradan. By ringing apple stock above and below the treated leaves Thomas and Bennett demonstrated that the phloem is the main conductive tissue involved in the transport of schradan (216). Tietz also ringed stems of *Pelargonium* so that the phloem was interrupted and found the translocation in the phloem interrupted and the translocation more than halved as compared with nonringed plants (219); the ratio of xylem to phloem movement was 1:2.2. Tietz obtained in his studies with radio tagged demeton indications that this systemic insecticide does not emerge from the elements of xylem and phloem by first moving to the end of the conducting bundles, but found lateral transference of compounds containing $P^{32}$ to the surrounding parenchymatous cells to a considerable extent. The path taken by demeton to pass from cell to cell is, according to Tietz (219) through intercellular spaces in the cell walls. Due to the lipoid solubility of the demeton, a portion is extracted by the protoplasma from these submicroscopic paths. Demeton is stored in the cytoplasm and when this is saturated, in the vacuols. The micro-radiographs of Tietz show clearly after application of radio tagged demeton large concentrations of $P^{32}$ compounds in the cells of the connecting bundle parenchyma and in the epidermal cells.

Differences in the translocation of the two demeton isomers were discovered by Metcalf *et al.* (152). The thiono isomer was localized in the oil glands because of its greater lipoid solubility. In fact very little thiono isomer is translocated, but the translocation is effected as a more soluble less stable metabolite and Metcalf found some indication that the thiono isomer may in part be isomerized into the thiol isomer before translocation occurs. This evidence corroborates the work of Heath *et al.* (95), but he believed that the thiono isomer contributed little or nothing to the systemic behavior of demeton, while Metcalf *et al.* showed that the thiono isomer is metabolized into an effective systemic insecticide. The slow absorption and metabolism of the thiono isomer is thus of definite importance in producing a persistent systemic insecticide (152).

There is evidence that systemics after leaf application are translocated with the assimilates, but the inadequacy of translocation downward is materially different from the behavior of sugars. In view of the complexity of the translocation phenomena in plants in general, it is not surprising that our understanding of the translocation of systemic insecticides is as yet confused. If translocation of systemics is based on a gradient of concentrations shown by Mason and Maskell for sugar, then storage or metabolization on the site of translocation would be necessary to maintain the concentration gradient.

The most important single factor in promoting translocation would appear to be light. There are differences in the behavior of different plant species, possibly dependent on the fact that in some plants active photosynthesis and in others products of this process are necessary for the translocation of systemic insecticides (216). Temperature is also an important factor in translocation promoting the transport of organic molecules in the phloem (231).

For the practice of pest control the effect of weather conditions on systemic insecticides is important. We have seen that in the case of the thiol isomer of demeton a rapid absorption removes that part of the systemic insecticide which does not evaporate from the plant surface but it would be wrong to assume that this removal protects it from losses by rain and wind. The rate of loss of systemic insecticides from inside leaves in the gas phase has been demonstrated and was found to be in good agreement with that expected on a simple physical theory. Systemics used for leaf applications are not very volatile and are much less volatile from dilute aqueous solutions. In plant species which guttate under normal conditions, systemics are freely excreted and this excretion can be more rapid than decomposition when the concentrations of the systemic in the plant are high, but it seems to be less rapid than decomposition at low concentrations (167). Under extreme conditions of wind and rain a considerable reduction of the persistence of the absorbed systemic insecticide was observed when Tietz compared the concentrations of $P^{32}$ marked demeton in plants exposed (a) to static air in the greenhouse, (b) to circulating air in the greenhouse, (c) to artificial heavy rain, and (d) to outdoor conditions. Under normal greenhouse conditions the concentration was linear up to the twelfth day when it had dropped to 0.010 milligram demeton per gram of plant and then slowed up. Under "windy" conditions there was a sharp drop in which the residual concentration of 0.012 milligram per gram was reached in 3 days, whereafter the remaining decrease ran parallel with that of the still-air plants. Under "heavy rain" the concentration fell in 3 days to less than 0.005 milligram per gram plant; the demeton was thoroughly washed out of the plant, in a similar way as nutrients are removed by rain out of plants (219). These experiments are in close agreement with practical experience on hops in England, when under conditions of persistent wind or heavy rain the aphis control with schradan spraying sometimes breaks down. It has therefore become the practice in periods of windy or rainy weather to increase the concentration of systemic sprays and revert to shorter intervals of application.

In conclusion, schradan, demeton, methyl demeton, Disyston®, the Fostion® EP *N*-monoisopropylamide of *O,O*-diethyl-dithiophosphorylacetic acid (190), monofluoroacetamide (213) have been shown to be useful systemics for foliar application.

## B. BARK APPLICATION

Jeppson's discovery (119) that bark treatment was more efficient than soil applications was confirmed by Bond (19) who worked in the tropics on coffee trees against *Planococcus kenyae* (Le Pelley) and obtained data indicating that the same degree of mealybug control could be effected with half the quantity of dimefox necessary for soil application. On cocoa in the Gold Coast banding with dimefox dissolved in agar on cocoa trees against *Pseudococcus njalensis* (Laing) was successful to get insecticidal quantities into the tree but did damage to the bark. Grob found that the bark treatment required 2–5 times the quantity of isolan adequate for good aphis control by foliar sprays; but he obtained a longer persistence and ecological selectivity (81). β-Fluororethyl fluoroacetate and demeton were effective against the pear aphid, *Toxoptera piricola* (Matsumara) in Japan (67), while demeton, schradan, dimefox and β-fluoroethyl fluoroacetate failed against the citrus scales, *Ceroplastes rubens* (Maskell) and *Unapsis yanonsis* (Kuwana). Seki *et al.* (199) in Japan obtained good control with demeton and schradan by bark treatment under bandages against red spider mites and obtained high mortality on the schradan treated plants a few days later than after demeton treatment. Sekitani *et al.* applied systemics successfully to hop stems and soya bean stalks (200).

The bark application method has advantages in reducing the health risk to operators and in producing selective action which might partially compensate for the greater cost of application on deciduous fruit trees, citrus, coffee, cocoa, and ornamentals. Volatile systemic insecticides require a covering while nonvolatile compounds can be applied simply by brushing or spraying the tree stem; this is practicable where small numbers of trees are treated, for instance in peasant agriculture. But for larger acreages mechanical sprayers actuated by breakerbars which operate a valve are feasible. As there are few anastomoses of the xylem, it is necessary to treat the complete circumference of the trunk. No systematic work seems to have been done on the type of bark suitable for this treatment and it is not clear whether systemics will go through dead bark.

### C. SOIL TREATMENT

The early work (177,182) has been confirmed by McLeod (145), Petty and Lochner (170), Emery (62), Fukuda *et al.* (67), Seki *et al.* (199), David and Gardiner (43), Sekitani *et al.* (200), Nomura (162), Jit Singh Verma (225), and Hanna *et al.* (85). Active ingredients of systemic insecticides have affinity to substances with active surfaces and are there absorbed. While the roots therefore freely absorb schradan, dimefox, or demeton from solutions, they absorb less from pure sand and even less from soil rich in colloids (David (39,40) Tietz (219)). Hence heavy clay soil or soils high in organic content require higher dosage rates for soil application of systemic insecticides than sandy soils. Four to eight times as much systemic is required for soil treatment as for trunk implantation to obtain the same concentration in the foliage, and it is thought that in addition to the abovementioned absorption to soil particles, microbiotic decomposition, leaching, and evaporation are responsible (85,86).

The absorption of systemic insecticides by the roots shows considerable variation. David found that roots of the broad bean plant selectively absorb dimefox from solutions (40) at rates of transpiration at which schradan was selectively rejected (39). Demeton is stored in the root bark and initially a far higher concentration is found in the root than in the shoot above, and finally the concentration in the root is greater than in the outside solution. Two processes seem to be involved in the entry into the roots. The first is a passive movement of the systemic in the transpiration stream through the roots and into the shoots. The second is an accumulation observed with dimefox and demeton to values higher than that in the surrounding solution. This form of uptake occurs in detached roots and independent of transpiration. Tietz (219) explains this phenomenon by the ultra filter lipoid theory and suggests that the lipoid solubility of demeton causes the latter to be absorbed in the lipoids in the root, after the compound has penetrated the wall of the root hairs. The uptake of demeton is similar to the uptake of antibiotics in higher plants, described by Crowdy *et al.* (34). Roots of Phaseolus plants immersed in a demeton emulsion are more permeable to water than roots placed in a check solution, and Tietz (219) explained the increased permeability by the assumption that the respiration is intensified.

Systemic insecticides absorbed by the root are transported to parts of the plant growing above ground in the transpiration stream in the xylem of the shoot axis and leaves in the same manner as nutrient salts. There is some lateral diffusion although within moderate limits (85,219). Therefore a circular application to the soil, root crown, or stem is necessary if insecticidal quantities of systemic insecticides are to be translocated to all aerial parts of the plant (19,85).

When the plant derives the systemic insecticide from the soil the systemic insecticide is accumulated in the peripheral portions of the leaves which act as blind ends in the parenchyma of the transpiration stream; phytocidal effects start, therefore, always on the edges of leaves.

The ability of plants to translocate systemic insecticides from the roots has been exploited for control methods because (a) a longer persistence of the systemic effect is obtained than with any other method; (b) certain systemic insecticides offer ecological selectivity while foliar application leaves them nonselective (182); (c) it is possible to treat plants before they have a lot of leaf, at a time when the surfaces which could absorb the systemic are confined to the stem (62); (d) acute toxic hazards during application are reduced as the atomization of the liquid is avoided (208).

Comparative studies have shown that dimefox, methyl demeton, demeton, dithiodemeton (19639 and Ekatin®), 3911 (Thimet®), and

Fostion® EP are all good soil systemics but that schradan gives a poor result when applied to the soil and mipafox has an intermediate position (190). Some authors have claimed good effects with schradan by soil application, such as Seki *et al.* (199) who compared demeton, schradan, and sodium selenate against *Paratetranychus citri* (McGregor) on potted citrus plants and obtained three months' protection. Nomura also had good results with schradan on egg plants and melons against red spider mites and cotton aphis but found soil treatment less effective than foliar sprays (161). Fukuda *et al.* compared the persistence of soil trench treatment on brassicas against *Myzus persicae* (Sulzer) using demeton, dimefox, schradan, and methyl demeton; dimefox and demeton gave the longest persistence but methyl demeton and schradan protected the plants for 36 days (67).

As to the best place for the soil trench treatment Hanna, Judenko, and Heatherington (85), Bond (19), and Jary (117) agree that the most economic results are obtained if the root crown is well wetted. In an experiment with dimefox to find the best technique against *Pseudococcus njalensis* (Laing) on cocoa, application at four equidistant spots 1 ft. from the trunk of cocoa trees was compared with application to circular depressions around the trunk at a distance of 1 ft. and 2 ft., and a circular depression around the root crown (85); the latter was found most effective which was confirmed by Bond (19) on coffee trees.

The effective dose was related to the weight of the tree by measuring and weighing cocoa trees which were cut out during the swollen shoot cutting out campaign. Hanna *et al.* (85) found empirically a correlation between the girth of the cocoa tree and its weight, and established a relationship between the dimefox doses which were lethal to the cocoa mealybug, *Pseudococcus njalensis* (Laing), and the girth of the tree; they call this the girth-weight correlation.

One treatment with dimefox against the cocoa mealybug had considerable persistence; the mealybug population build-up after 6, 12, 18, and 24 weeks was 0.08, 2.5, 9.4, and 15.5% of the population before treatment. One treatment kept the trees toxic for about 7 weeks and repetitions at 8-week intervals kept the population at a very low level for many months (85).

On hops, Emery (62) and Jary (117) have confirmed our earlier work (182) that soil application of dimefox gives good hop aphis control, and two months' control of hop aphid is also reported after soil trench with Thimet® (55). Thimet® has also been successful against other aphids, thrips, and certain Lepidopterous larvae (*Pieris rapae*). Soil texture, moisture, microbiology, as well as soil conditions interrelated with the variations of temperature, humidity, and sunlight, increase the complexity of the factors influencing the absorption and translocation, but Hanna *et al.* (85) found that heavy rains in the tropical rain forest had little if any influence on the treatment with dimefox, even though dimefox is subject to leaching; considerable quantities were found in nearby creeks after heavy rainfalls.

Soil application of dimefox has been satisfactorily used in England on Brussel sprouts, sugar beets and mangolds to control aphids. In Japan, demeton, schradan, dimefox, and β-fluoroethyl fluoroacetate applied as a soil trench to cabbage seed plants kept the crop free until harvest and did not affect the quantities of seed harvested from these plants (67). Good results against citrus aphis (*Aphis citricidus* Kirkaldy) and citrus red mite *Paratetranychus citri* (McGregor) are also reported with monofluoroacetamide and β-fluoroethyl fluoroacetate. In a comparison of demeton, dimefox, schradan, and β-fluoroethyl fluoroacetate, the first two were found the best against *Myzus persicae* (Sulzer) on brassicas (67).

While translocation upward can be relied on to occur in the growing season, experiments to kill root aphids have been less successful.

There are several methods of applying systemics to the soil used in pest control, which are sometimes described as soil trench treatments.

A continuous jet spray onto the row at the base of the plants by conventional row crop sprayers is widely used. For intermittent soil treatment aimed at distant planting stations, mechanical soil trench applicators have been used experimentally, whereby the discharge mechanism was actuated either by a touch rod contacting the plant, or by a selenium cell. Manual application to planting stations is carried out by a spoon or manual soil injectors. Mechanical soil injectors using metering pumps discharging through an open pipe behind cultivating tines close to plant rows are also used.

Simultaneous applications with the seeding operation have been developed; in a recent refinement of this method the seed is placed by a conventional precision drill; a certain amount of soil is then placed above the seed by only half filling the furrow cut by the drill. The systemic insecticide is then applied in a 2-inch band into the furrow, and a drilling chain closes the furrow by dragging in soil from the sides before the soil is consolidated by a roller (180).

Systemics may also be added to the transplantation water of conventional transplanting machinery during planting out (174).

Experiments to apply systemic insecticides in irrigation water in the field failed. The placing of accurately determined quantities of systemics in gelatine capsules which dissolve in the soil moisture has also so far proved impractical because of the frequent occurrence of leakers after ageing of the capsules and after exposure to extreme temperatures. Small charges of systemics have been attached to wire and covered with water-soluble substances for insertion in the soil (capsticks) and this has proved a simple application method for ornamentals which avoids toxic hazards to operators.

Most applications of systemics to the soil have hitherto been made as water solutions because earlier experiments to apply systemics absorbed on carriers proved less efficient.

Thimet® is now supplied also on granular carrier containing 2.16% for side dressing (placement) or broadcasting against sugar beet maggot, leafhoppers, aphids, Lygus-bugs, fleabeetles and wireworm, cabbage aphids, cabbage worms and maggots, bean beetle, seed corn maggot, mites; recent research has shown that systemics applied in granular form are more effective than soil trench treatment (147).

Soil applications are obviously safe for seed crops, ornamentals, and forest trees, but a careful study of application to food crops is necessary to ensure complete decomposition by harvest time, as soil application introduces factors additional to speed of decomposition in the plant; the growing roots later in the season tap further soil areas containing the systemic, or the latter is redistributed through rain or irrigation water and taken up too close to harvest time to be completely enzymatically decomposed.

Although soil treatment is generally much more expensive than foliar application there are cases in which this disadvantage is outweighed, because the soil application method prevents damage to wild life and game birds, reduces application costs, prolongs persistence of the toxicity of the plants, and achieves ecological selectivity (134).

## D. TRUNK IMPLANTATION

In order to eliminate several sources of loss in the methods previously described, the implantation of a systemic insecticide into trees under antiseptic conditions suggested itself. After brushing of the bark on the sites of the projected implantation with a disinfectant, holes were cut by a pneumatic drill at an angle of 45° to the main axis of the tree to a standard depth of $3^1/_2$ (86). The filings were blown out and dimefox injected by a converted veterinary syringe. The borehole was chosen so that the required volume of the systemic solution nearly filled the hole which was then sealed by slightly tapered wooden or metal bungs with a coarse thread. For re-treatments some metal bungs had a central hole into which a removable screw was inserted. After completion of the implantations, the bungs were covered with tree wax. No fungoid or bacterial infections of the tree were encountered but there were discolorations in the wood above the borehole and a considerable exudation from the holes continued for some time. With higher dosages of dimefox, some shedding of leaves of the cocoa trees was observed but the trees were bearing normally and the life of the trees was not affected. As Hanna, Heatherington, and Judenko (85) found the greatest reduction of cocoa mealybugs on branches projecting directly above one implantation hole while no reduction was found from branches originating from the trunk opposite to the borehole, and an intermediate effect on branches originating in intermediate positions, the necessary number of holes for bearing cocoa trees was determined in a number of experiments as 4–5. Translocation upward and downward was measured by counting mealybugs from branches originating from the trunk above and below the boreholes on trees which had been injected 3 weeks previously with 1–2 grams dimefox in 10 cc. water. There was a considerable reduction of mealybugs above the injection hole but next to no reduction below. Hence the location of the hole at soil level was considered optimal.

Hanna *et al.* implanted fractions of dose required for soil application according to their girth-weight correlation but obtained thus too high a dosage for small trees while big trees were underdosed, and therefore concluded that the dimefox dosage for trunk implantation should be calculated in p.p.m. of the tree weight; 160–250 p.p.m. were found to give an excellent control of the cocoa mealybug corresponding with that obtained by the soil treatment, thus effecting a substantial saving over soil application, i.e., 75% and over of dimefox in the case of a tree of 10-inch girth.

At present the trunk implantation method is laborious but could be quickened by mechanization; an alternative simple method has been proposed in the form of so-called "tree nails" which are large nails which carry in the concave cap with a diameter of about $^3/_4$ inch a dose of the systemic insecticide absorbed in a gelatinous substance which is conveyed by a lumen in the spike of the nail into the tree, or relies on uptake by the bark. This method is under test for forest and shade trees (Caughey and Bond (29)).

### E. SEED TREATMENT

This method is the realization of one of the oldest dreams of farming men, which according to a seventeenth century text was thought of as early as A.D. 50 by Junius Columella (David (46)). The earlier claims (177) to render plants insecticidal by translocation of systemic insecticides applied to the seed has been confirmed (53, 111,113). David and Gardiner (46) have shown that, using large seeds, the soaking in water solutions of schradan, dimefox, paraoxon, sodium fluoroacetate, and demeton produced plants which are toxic to aphids but that this effect is difficult to achieve with small seeds. Emulsions of demeton were absorbed by broad beans in equal quantities with water. Beans vary greatly in the rate at which they absorb solution, so that a period of 24 hours' soaking was necessary. The insecticidal property of a plant growing from a treated seed is directly related to the quantity of solution absorbed by the seeds, and as larger beans absorb more solution than small beans the plants grown from them are more toxic. After short periods of soaking (4 hours) there was more insecticide in the seed coat than in the cotyledon, but after 24 hours the position was reversed; systemics stored in the cotyledon can pass directly to the growing plant. Seed soaked in the insecticide, dried and stored for one month produced plants toxic to aphids. As was expected, the same quantity of demeton was found to be more active when absorbed by a seed than when watered onto the soil around it (46).

As small seeds do not take up enough of the systemic insecticide from the solution to protect the young plant, other workers (111) treated seed with solid absorbents impregnated with systemic insecticide, because this method is obviously equally applicable to large seeds. Recently phosphorodithioates (Thimet® and Bayer's Disyston®) have been used as seed treatment (31,183) as 44% on activated carbon with 2% methyl cellulose at such a rate as will give

2–4 lb. per acre. This has given protection on young cotton plants, vegetables such as peas, beans, and sugar beet for 3–7 weeks against thrips, aphids, chrysomelids, leafhoppers, wireworms, red spiders, eriophyid mites, seed corn maggots (Anthomoyids) (5,111,173), cotton leaf perforator (*Bucculatrix thurberiella*), the cotton fleabeetle (*Podagrica puncticullis*) and cutworms (147,183).

On Thimet treated cotton, temperature, and soil moisture influenced germination and stand. While good conditions (75–80°F. and soil moisture up to 30% of field capacity) produced good germination and stand, cool weather (60–65°F.) and wet conditions (60–70% field capacity) caused a decrease in stand (5), but Bayer's Disyston® is claimed to be safer even under unfavorable conditions.

Kantack (121) obtained a uniform plastic-like coating of the seed with carbowax 6000 (a water-soluble polythene glycol). The systemic insecticides were added to the molten carbowax in the desired proportion and the seeds were kept in motion while this formulation was poured over them. Schradan, demeton, methyl demeton, and American Cyanamid's 12008 and 12009 were compared. Good germination was obtained when $^1/_2$ lb. or less of the toxicant was blended with 4 ml. carbowax 6000 per bushel of seed. This seed treatment achieved a satisfactory reduction in the population of the wheat curl mite, *Aceria tulipae* Keifer, and was superior to the soil trench method during the first four weeks; the superiority of the seed treatment was most noticeable during the first week, while the soil trench method was found to be more effective during periods of rapid growth and after four weeks (121).

The seed treatment method is filling an important gap in crop protection practice which arises when early pest attacks occur before the plant has enough foliage to hold and absorb sufficient spray, or when virus infections by aphids are expected on very small seedlings, such as in the case of sugar beet stecklings grown in summer for steckling production in yellow virus areas in Europe, or in the case of autumn-sown sweet pea seeds threatened by pea mosaic virus.

## III. Practical Uses of Systemic Insecticides in Pest Control

Systemics have meant a great advance in the control of inaccessible pests secreted in crinkled leaves or even galls (aphids), in the leaf tissue (Eriophyidae and leaf eelworms), under bud scales (red spiders and Eriophyidae), even under tents built from debris by symbiotic ants over mealybugs, or hidden away in cracks (aphids and mealybugs), and finally just protected from the spray by sitting on the underside of the leaves. As there are less survivors of the systemic treatment in hidden positions, resurgences where they occur with nonselective systemics have to start from smaller foci and consequently less treatments are required than with ectotherapeutical compounds.

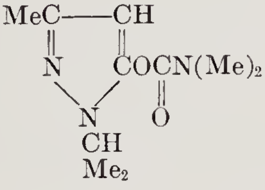
The reality of the claim that young foliage developed after treatment with systemic insecticides will be toxic, has sometimes been disputed. After soil application and trunk implantation, new foliage will be rendered toxic after treatment for a considerable time, but with foliar application, the amounts which are translocated to foliage developed after the systemic treatment depend on the dosage and also on the systemic's half-life in the plant. Many failures of translocation have been caused by determining the dosage on mortality counts only, without reference to the phenomenon of translocation to other leaves, and where manufacturers have tried to show economic advantages by setting their recommendations low, insufficient translocation to later developed foliage occurs.

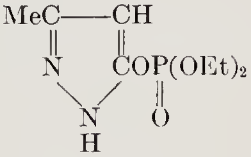
Turning to the range of pests controlled, a large number of papers testify to successful control of aphids with schradan, demeton, methyl demeton, Thimet®, Fostion® EP, mipafox, Isolan, or 1-isopropyl-3-methyl pyrazolyl-(5)-dimethyl carbamate (XV) pyrazoxon or *O,O*-diethyl *O*-(3-methylpyrazolyl)-(5)-phosphate (XVI), Phosdrin® and monofluoroacetamide, too extensive to review (4,35,37,38,67,73,75,79, 104,114,116,122,145,155,156,161,163,169,171,176,183,203,204,208,215, 223,234,235).

Schradan gives the most persistent toxicity to aphids, followed by demeton, while methyl demeton takes an intermediate position, and Phosdrin® gives excellent toxicity of a very transient nature, useful for crops to be harvested shortly afterwards, e.g., for the control of *Myzus persicae* well secreted in lettuce plants.

Commercially schradan is used on a large scale in England on hops against *Phorodon humuli*, at a rate of 12 oz. of Pestox 3® containing 55% of the active ingredient to 100 gal. as a spray applied at the rate of 100–250 gal. per acre; further on various Brassica crops against *Brevicoryne brassicae* at the rate of 2 lb. schradan per acre; against *Aphis fabae* and *Myzus persicae* at the rate of 2 lb. per acre in 30–100 gal. as an over-all spray, or as a band application in the period between singling and before the sugar beets cover the ground, at the rate of 0.75 lb. in 10–15 gal. per acre; at the same strength on strawberries against *Pentatrichopus fragariae.* In the United States schradan has had some application against *Aphis gossypii,* against which pest it is also used in the Sudan at 0.5 lb. schradan per acre applied in 2 gal. Schradan has been very successful in California against walnut aphis, *Chromaphis juglandicola* (155a and b), at 1–4 lb. in 100–200 gal. per acre.

Demeton is used on a large scale commercially in the United States, in Europe, and parts of Africa, against aphids on apples and pears (0.19 lb. technical material to 100 gal.); the same strength against aphis on potatoes and at 0.38 lb. against aphids on strawberries and musk melons; while 0.5 lb. per acre is used against *Brevicoryne brassicae;* at the rate of 0.37 lb. per acre in 25 gal. for ground spraying or in 1 to 2 gal. for aeroplane spraying, it has been widely used in the United States and in the Sudan against *Aphis gossypii.* Other commercial uses of demeton include citrus where it is used as Systox® at a dosage of 4–8 oz. per 100 gal. against *Aphis spiraecola* and *gossypii,* further against aphis on beans, seed, alfalfa, sugar beet, nonbearing deciduous trees, and pineapples.


(XV)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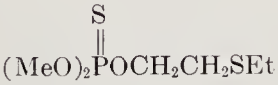
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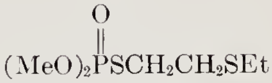
(XVII)


$(\mathrm{MeO})_2\overset{\overset{\mathrm{O}}{\|}}{\mathrm{P}}\mathrm{SCH_2CH_2SEt}$

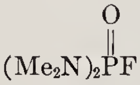
(XVIII)

Methyl demeton is used widely as Metasystox®, a 7:3 mixture of *O,O*-dimethyl *O*-2-ethylmercaptoethyl phosphorothionate (XVII) and *O,O*-dimethyl *S*-2-ethylmercaptoethyl phosphorothiolate (XVIII). Dr. Schrader, the discoverer of many systemics including Metasystox®, has recently developed a new process which gives a high percentage of the more useful thiol isomer and a new formulation principally consisting of this isomer is marketed as Isometasystox.® Methyl demeton is used in Europe against the same range of pests as demeton at roughly twice the dosage (189).

A soil trench treatment against hop aphids is becoming quite widely used in Britain and in Belgium in hop gardens where dimefox (XIX) as Hanane® containing 50% active material is used at the rate of 1 quart in 25 gal. per acre at the rate of 4 fluid oz. per hop crown. A single application gives seasonal protection and should not be made within 8 weeks of harvest.

Where no residue problems arise, systemic insecticides are now the preferred method of aphis control for all but root feeders.

Schrader's experiments with foliar sprays and soil application of bis-(2-fluoroethoxy)-methane (XX) resulted in *Phylloxera* control but caused toxic residues (Martin and Shaw (140)). It has been reported earlier (177) that infestations of *Pemphigus auriculae* are readily killed, but extensive experiments undertaken in New Zealand (116), Germany (76), and Italy failed to obtain a good control of the *Phylloxera* on grapes. Dimefox, schradan, demeton, and other systemics were used but none of them reduced the *Phylloxera* infestations on the roots presumably because translocation of the systemics to the roots is inadequate. Zanardi reported recently that foliar sprays and soil applications carried out in a vineyard near Lake Garda in Italy with demeton at six times normal dosage rate and with schradan at four times normal dosage rate achieved no control of *Phylloxera* infestations on the root and was followed by no recovery of the general health of severely affected plants (240).



$$(Me_2N)_2\overset{\overset{O}{\|}}{P}F$$

(XIX)

$$H_2C(OCH_2CH_2F)_2$$

(XX)

Systemic insecticides have shown great promise against certain scales, particularly soft scales and mealybugs. A control of *Lepidosaphes ulmi* (L). was obtained with demeton by root application and by foliar treatment (197). Still better control is obtained with dithiodemeton or Disyston® and in some countries by Ekatine® which is effective against red scale (*Aonidiella aurantii*, Maskell), citricola scale (*Coccus pseudomagnoliarum*), cottony cushion scale (*Icerya purchasi*, Maskell), soft scale (*Coccus hesperidum*, L.) (175). Tetram® *O,O*-diethyl *S*-2-diethylaminoethyl phosphorothiolate (XXI) shows promise against the scale insects and shows a long lasting systemic action (150). In Japan a high mortality of red wax scale, *Ceroplastes rubens* (Maskell) followed foliar application with monofluoroacetamide (67,114,160) and β-fluoroethyl fluoroacetate. Although these fluorine compounds were least effective against the third instar and adults of the citrus mite, *Unaspis yanonsis,* Fukuda *et al.* (67) and Iyatomi (114) obtained nearly complete control of the first and second instar of this scale.

Mealybugs were controlled with dimefox, for instance the already mentioned cocoa mealybug (85,86,158), by *Pseudococcus brevipes* on pineapples in Hawaii and South Africa (4) and others (116). Dimefox tried on coffee against *Planococcus kenyae* (LeP.) while effective during certain periods of the year (19), was much more expensive than the control of the *Pheidole punctulata* (Mayn); without the protection of this ant, the mealybug is controlled by the parasite, *Anagyrus kivuensis* (Compere). Dimefox was effective in the control of a psyllid (Nicol (159)). There is general agreement that whiteflies are not adequately controlled by schradan or demeton, nor by Disyston® (183).

As far as leafhoppers are concerned, some investigators report control by systemic insecticides. For *Empoasca fabae* (Harris) good control has been achieved with demeton (53,146) and with schradan by McLeod (145). Dithiodemeton was effective against the beet leafhopper (*Circulifer tenellus,* Baker (101) as Thimet® (5) and demeton (150). Hutchinson obtained four months' toxicity against the blunt nosed leafhopper by soil trench treatment of potted cranberry plants with demeton (110). For *Empoasca lybica* (deBerg) on cotton in tropical Africa methyl demeton applied at the rate of 0.25 lb. per acre gave control for 7 days (183).

Many good results of control of thrips with systemics have been reported (29). Demeton was effective against *Taeniothrips simplex* (Mor.) (230), schradan, demeton, and methyl demeton against *Frankliniella fusca* (Hinds) (116), as was Thimet® and its isopropyl analogue as seed treatment on cotton against *Franklinella fusca* and *Thrips tabaci* (5,150).

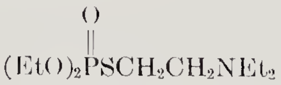
Böhm (14) reported control of the larvae of sawflies, *Hoplocampa testudinea* (Klug) and *H. brevis* (Klug) and *Neurotoma flaviventris* (15), with methyl demeton but dithiodemeton seems to give a still better control of saw fly larvae.

Demeton, methyl demeton, and schradan have also been successful in killing leaf miners. *Pegomyia hyasogami* (Panzer) on sugar beet and mangolds is controlled by demeton (236) and methyl demeton

(56), *Phytomyza atricomis* (L.) by schradan on chrysanthemums, as well as *Liriomyza langei* (Frick) with demeton (236). Good control of *Dasyneura affinis* by soil treatment with schradan and foliar application of schradan, mipafox, and demeton has been reported by Böhm in Austria (18).

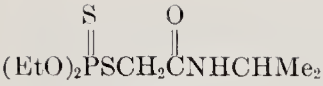
Good control of the olive fly, *Dacus oleae,* and cherry maggot, *Rhagoletis cerasi* L., is achieved by the *N*-monoisopropylamide of *O,O*-diethyldithiophosphorylacetic acid (XXII) Fostion® EP, the *N*-monomethylamide of *O,O*-dimethyldithiophosphorylacetic acid (XXIII) Rogor® and the *N*-monoisopropylamide of *O,O*-dimethyldithiophosphorylacetic acid (XXIV) (Fostion® MP) (50,51,168). There would appear to be little difference in the insecticidal performance of the three compounds against larvae of the Trypetid flies, but the toxicity to mammals of *O,O*-dimethyl derivatives in lower than that of the *O,O*-diethyl derivatives; although these compounds have good systemic action against red spiders and aphids (183, 190), it is probable that they function in the control of the *Trypetid* larvae as penetrating insecticides only. Demeton and schradan prevent a larval infestation of *Rhagoletis cerasi* (L.) developing from laid eggs for 20 days (Schuhmann (198)).

There are records of control of leaf-eating insects by systemic insecticides, particularly demeton, methyl demeton, Thimet® and dithiodemeton, but in some cases contact or vapour toxicity and systemic effects are involved (44). David and Gardiner (44) have applied demeton to the root of plants and found it to act on all stages of *Phaedon cochleariae* (F.) (except eggs) and *Pieris brassicae* (L.), but compared with the dose which killed aphids, about twice as much was required to kill *Phaedon* larvae and 20 and 200 times as much to kill *Pieris* larvae; when applied systematically, demeton like paraoxon can prevent the emergence of *Pieris* larvae from the eggs. Thimet® and dithiodemeton are effective against the cotton leaf-perforator (*Bucculatrix thurberiella*), the cotton flea beettle (*Podagrica puncticollis*), cutworms, and other lepidopterous larvae (5,147,183); Phosdrin® is also very promising against the latter. It is also established that flea-beetles are controlled on vegetable plants grown from Thimet® treated seed (5).



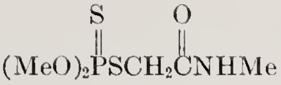
$$(EtO)_2\overset{\overset{O}{\|}}{P}SCH_2CH_2NEt_2$$

(XXI)



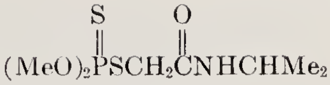
$(EtO)_2\overset{S}{\overset{\|}{P}}SCH_2\overset{O}{\overset{\|}{C}}NHCHMe_2$

(XXII)



$(MeO)_2\overset{S}{\overset{\|}{P}}SCH_2\overset{O}{\overset{\|}{C}}NHMe$

(XXIII)



$(MeO)_2\overset{S}{\overset{\|}{P}}SCH_2\overset{O}{\overset{\|}{C}}NHCHMe_2$

(XXIV)

Many Tetranychid mites are well controlled by foliage sprays with schradan (52), demeton (115,205), Thimet®, Fostion® EP, Tetram,® (59,77,78). Pyrazoxon (208), Phosdrin® (32), and methyl demeton (22,79,116,122,145,161,188,199,200,214) also monofluoroacetamide (114) and $\beta$-fluoroethyl fluoroacetate (67); by soil application dimefox has given good results (4).

Wherever combined red spider and aphis control is required, systemic insecticides are the preferred method (54,156). Good control of eriophyid mites has been obtained (16,54,145) and this method has obtained a permanent place in pest control practices (129).

The effectiveness of demeton, schradan and ectotherapeutical acaricides was compared by Henderson and Tilton (99) against the brown wheat mite, *Petrobia latens* (Müller); demeton at 0.5% and 0.25% was found better than schradan and these systemics were among the best of all the insecticides tested.

Foliar nematodes have been controlled with several systemic insecticides. Against *Ditylenchus dipsaci* (Kuehn), Feder (64) reported 100% control in leaves and 93% in the bulbs of daffodils by applying a soil trench spray with demeton around the basis of the plant. Foliar applications were less effective. Sasser (192) reduced root knot nematodes on tomatoes and cucumber by demeton soil treatments, but the concentration to effect a 100% mortality was too phytotoxic. Sher, Kamasaki, and Murakishi (202) obtained with diethyl-1-chlorovinyl phosphate (OS 1836) and demeton 100% control of *Aphelenchoides besseyi* on Vanda-orchids. *A. fragariae* (Ritzema Bos) on fern was inadequately controlled by a single application of demeton, but four applications with 0.05% solution of Systox® in 3- to 4-day intervals gave control (Hirschmann (102), Böhm (17)). The same repeated treatment gave control of *Aphelenchoides ritzemabosi* (Schwartz) (102) and laboratory tests against *Ditylenchus dipsaci* (Kuehn) indicated that this foliar eelworm might be controlled in a similar way. Highly significant control of this species on alfalfa was obtained by Bergeson (13) with demeton and on alfalfa and daffodils with diethyl-1-chlorovinyl phosphate, OS 1836; three spray treatments were necessary to obtain complete control. In case of daffodils, nearly 100% control was obtained by applying demeton and OS 1836 as a drench at concentrations as low as 0.1%. This degree of control was obtained with OS 1836 within one week and with demeton in four weeks. Some phytotoxicity was observed with OS 1836 and demeton at 1.0% and 0.5%. Schradan gave substantial control but was not so consistent as the other two systemics (13).

In Japan experiments with demeton in the dipping liquid of rice transplants for the control of *Aphelenchoides oryzae* were successful, while field treatments with demeton were followed by some reduction but did not achieve control.

## IV. The Prevention of Virus Spread by Vector Control with Systemic Insecticides

Because of the better control obtained and the susceptibility of many important virus vectors to this group of insecticides, systemics were early suggested for virus prevention by vector control (181). It is obvious that virus diseases could be prevented simply were there systemic insecticides available which killed the virus-infected vectors soon after they alighted on the crop to be protected and before the feeding insect could transmit the disease to the plant, but the systemic insecticides of today do not kill as fast as this (98,184).

Confirming previous observations of this type by Watson (228, 229), Dickson according to Reynolds (174) and Walrave (227) have shown that alatae of *Myzus persicae* (Sulzer) in which the period of circulation of the potato leaf roll virus had expired when flying into a noninfected field are able to infect numerous plants until killed there by the systemic insecticide. The virus infection of a healthy plant is accomplished in the feeding time of 2 hours, as against the 45 hours required to obtain 85% mortality with schradan and 15 hours to obtain the same mortality with demeton. As the period during which the vector becomes capable of transmitting the potato leaf roll virus is 48 hours, a spread of the virus by Aptarae and Alatae which arrived uninfected on a sprayed field can be prevented; such treatment also prevents the spread of the virus from foci on the field because aphids cannot propagate on plants treated by a systemic. Two demeton applications in German and Dutch seed growing areas considerably decreased leaf roll virus within the crop, but did not check the virus from infecting the crops from outside (100,185,186). Similarly in Switzerland demeton did not prevent the spread of viruses when aphids were plentiful (191,209). Klostermeyer's plot experiments in Washington gave similar results (125).

Broadbent, Burt, and Heathcote (21) have also demonstrated that it is possible to prevent the spread of leaf roll virus from infected to healthy plants by fortnightly application of either persistent contact or systemic insecticides, when applied at high volume sprays with overhead and underleaf cover. The control of rugose mosaic (virus Y) is more difficult, because aphids can acquire and transmit virus Y within a few minutes, before they are killed by the insecticide but spraying, nevertheless, decreases its incidence by reducing the number of vectors spreading the disease from infected foci. Broadbent *et al.* therefore concluded that the limiting factor is the amount of virus Y introduced (21). This introduction occurs mainly in July in the main potato growing areas of England, when Alatae leave the potato crop. To prevent this Broadbent *et al.* recommend in addition to repeated spraying of the seed crop that an aphicide be included with the anti-blight sprays of the potato crop grown for market in the area so as to maintain the healthy stock.

Where a low degree of virus infection is acceptable, the treatment with a persistent systemic offers a good economic return because it reduces the spread of the virus on the field; this effect is greatly enhanced if a persistent systemic insecticide is also used on the infected crops from which viriferous Alatae emigrate. A well explored example is furnished by yellow virus on sugar beet in which a low percentage of virus infection can be tolerated, and it is only necessary to delay the virus infection long enough to ensure that the loss of sugar yield caused is not of economic significance, i.e., in England until after midsummer (108). Much of the early increase of disease after the first infection of the field is due to spread within the field. Aerial observation of an untreated sugar beet field shows early in the season a great number of foci of yellow virus infection which spread in size as the season advances and finally coalesce. Timely spraying with systemic insecticides kills the aphids on the crop and delays the spread of virus. In parts of Western Germany spraying with systemic insecticides has been adopted as a routine measure. Area warnings to spray are given when the average number of aphids reaches 0.5–1 per plant and this treatment gives yield increases of 10% when yellows are mild (35,36,120,127,201,210,211).

Experiments conducted in England by Hull (107,108,109) have shown a halving of the virus infection or better giving yield increases of 8–10%; in one severe virus outbreak year 90–100% of the sprayed plants became ultimately infected, nevertheless, the yield of the twice sprayed plots increased from $10^1/_2$ to $14^1/_2$ tons of washed beet per acre. In the course of five years of such experiments Hull reported "reasonable responses" in 14 out of 60 experiments and therefore recommends that spraying should be delayed until aphids are found on the plants. A good effect was then obtained from one spraying, even in mild yellow virus years, and a second spraying gave additional yield increase although the yield response to two treatments is not so great as the sum of the increase of two sprays applied separately, but in years of severe outbreaks economic yield responses are obtained from two sprayings (108). Reductions in the virus infection were obtained in Austria by Wenzl and Krexner (233) but the incidence of the disease was so variable that routine systemic treatments are considered uneconomic (196).

Ernould (63) who has carried out similar experiments in Belgium from 1950–1955 comes to the conclusion that systemic treatments are justified in districts suffering regularly from medium sized and heavy attacks of virus yellows. In a great number of careful experiments conclusive proof was obtained that systemic treatments gave significant increases in root production, sugar content, and purity of sap and beet tops, while even repeated treatment by nonsystemic insecticides were without significant effect on yield. Systemic treatment of sugar beet fields might be insufficient in years where a great number of aphids and much virus source occur. Virus spread is therefore in England further restricted by prevention of emigration of viriferous Alatae from sugar beet seed crops and from stored fodder beet. On Hull's recommendation stecklings are only certified for transplanting if the virus infestation is less than 1%. This is achieved by growing beet seedlings under a cover crop of barley and spraying with systemics when the barley is harvested (107), which according to Hull and Gates reduces virus infestation to one-tenth (109). A cover crop was necessary because uneven germination in dry years made it difficult to time the first spraying and sometimes the seedlings were too small to retain enough spray chemical when great numbers of viriferous Alatae infected them. This is a case where soil and seed treatment with systemics fills an important gap in present methods of pest control because it protects the plants from their first emergence. In years of prolonged migration of viriferous Alatae from sugar beet fields to young steckling beets a very high percentage of infection occurred despite spraying with systemics. This can be overcome by correct siting of the steckling beds (109) away from commercial beet or a treatment of the commercial beet in the neighborhood with a persistent systemic prior to emergence of the steckling beet sufficiently decreased the influx of viriferous aphids; less than 1% of the stecklings were infected by viriferous aphids from outside before they were killed by the systemic treatment.

Sugar beet seed producers have found that virus control not only helps the beet growers but also substantially increases the seed yield by 25–50% (79,108).

Hull and his collaborators have also shown that fodder beets carry virus and aphids over the winter. When part of stored fodder beet has to be kept until into the spring, it is recommended that part of the field from which the fodder beet are to be kept until next spring, should be sprayed before harvesting with a transient systemic but this recommendation has as yet not become general practice.

A reduction in the incidence of a persistent pea virus from 53% in untreated plots to 20% was obtained with three foliage sprays with demeton by Swenson, Davis, and Schroeder (212), resulting in increased yield.

This concept to prevent virus spread has also been tested against the swollen shoot virus of cocoa in the Gold Coast; these tree viruses are enormously damaging and are transmitted by *Pseudococcus njalensis* (Laing), *P. citri* (Risso), and *Ferrisia virgata* (Ckll). The incubation period of the swollen shoot virus in cocoa is prolonged, namely from 6 months to $2^1/_2$ years depending on the strain of the virus. A roguing of the visibly infected trees which so far has been the practice of control always passes over adjacent trees which have the disease but show no symptom of it at the time of cutting out of the visibly infected trees. In the cutting out process, trees heavily infested with mealybugs may crash to the ground and shed a great number of viriferous mealybugs which on their own, or assisted by their symbiotic Crematogaster ants, may escape to nearby healthy trees and infect them. A systemic treatment of visibly infected trees before cutting out and of several rings of "contact" trees around them has therefore been suggested by the writer. The cutting out operation after the control of the mealybug by dimefox reduces the amount of infectious material without risk of releasing viriferous mealybugs, and the "contact" trees are kept for the incubation period mealybug-free by re-treatments with dimefox. During this incubation period the "contact" trees are inspected, and as the virus infection becomes manifest they are removed. Pest Control Ltd. have carried out a field-sized demonstration of this method on 13 acres on which dimefox was applied to

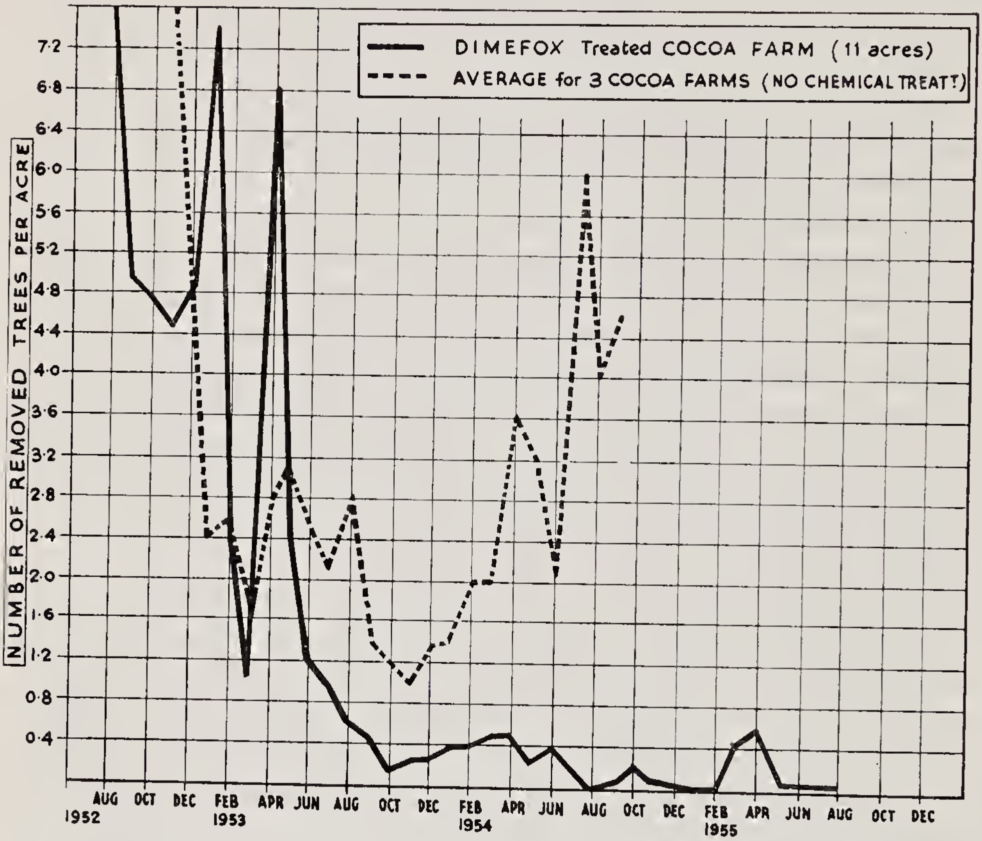


Fig. 1. Incidence of visible swollen shoot infection of cocoa trees after vector control with dimefox and removal of visibly diseased trees in comparison with removal of visibly infected trees only (Hanna *et al.* (84)).

the cocoa trees by the soil trench method nine times. The trees were examined and every one of the visibly infected trees was removed. The number of diseased trees fell very rapidly a year after the first treatment and the figures remained low up to the completion of the observations. Three cocoa farms in the neighborhood where the initial swollen shoot infection was lower at the commencement than on the dimefox-treated farm served as comparison, after the initial drop following cutting out the neighboring farms registered a rapid increase in the numbers of trees showing visible infections and a higher incidence of infection in the "control" farms continued to the end of the observations (Hanna (84)) (Fig. 1). At the date of writing, the dimefox-treated area is a beautiful stand, while on neighboring farms cutting out has had to be continued regularly and the stands have therefore become far from satisfactory. Dimefox is decomposed in the plant to such a degree that a nil residue is obtained in the cocoa beans; a concentration of dimefox in the sun-dried fermented beans not exceeding 0.2 p.p.m. as laid down by the Food and Drug Administration to be a nil residue (177). Although this treatment is effective and it constitutes an advance over the cutting out treatment by itself, it has so far not been adopted by the Gold Coast authorities.

While persistent ectotherapeutical insecticides have been used successfully against insect vectors which are not secreted (21,125) vectors in inaccessible positions can be controlled effectively only by a systemic insecticide (63). There are other important considerations in the choice of insecticides for vector control dealt with in the next section.

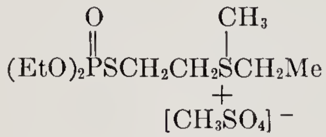
## V. Selectivity and the Integration of Biological and Chemical Control

In contrast with the contact toxicity of most of the other systemics now in general use, the physiological selectivity has only been proved for one systemic, i.e., schradan and has been the subject of further investigations (53,122). This phenomenon was originally discovered when schradan was used against aphids and their natural enemies (177,181,182) but the margin of selectivity may be different if representatives of very different groups of insects are concerned. Studies of the contact toxicity to the corn leafhopper, *Peregrinus maidis* (Ashm.) and the predacious Mirid bug, *Cytorrhinus mundulus* (Breddin) which lives on the eggs of the leafhopper, showed that schradan was about three times as toxic to the pest as to its predator, while demeton was equally toxic to both species, i.e,, a schradan concentration which killed 98% of the leafhopper, killed 20% of the Mirids (225).

Ahmed (1) examined the effect of feeding predacious larvae on cotton aphids killed with demeton and schradan, and confirmed our previous finding that aphids killed by schradan are not themselves toxic; aphids killed with demeton by the dipping method were toxic to *Coccinella septempunctata* (L) and to *Scymnus syriacus*, but both species survived when fed with aphids killed without external contact with demeton. Larvae of the Syrphid, *Sphaerophoria flavicanda* (Zett) and of the Ochthiphitid, *Leucopsis puncticornis* (F.) were killed when fed on demeton- and schradan-killed aphids, but *Chrysopa vulgaris* could stomach demeton- or schradan-killed aphids with impunity.

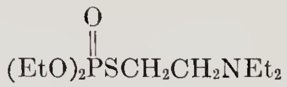
Several hypotheses have been put forward to explain the physiological selectivity of schradan, of which the two most likely will be reviewed: Basing themselves upon observations of the relative converting capacity of various tissues, O'Brien and Spencer (164) suggested that susceptible insects convert schradan to the cholinergic oxidation product slowly within the nerve tissue, while in the nonsusceptible species the conversion rate in the fat body of the insect is so great that little or no unconverted schradan reaches the nerve tissue and the short-life oxidation product of the schradan is incapable of penetrating the insect lipoid nerve sheath. Casida (25) and Casida *et al.* (27) believe that the susceptibility of insects to schradan depends upon the sensitivity of the particular insect cholinesterase to the inhibition by the schradan metabolite, and demonstrated that the cholinesterase from the nonsusceptible *Trialeurodes vaporariorum* (Westwood) and *Blattella germanica* (L.) required 6–24 times as much oxide of schradan for inhibition as that of the housefly.

There are two new compounds with an insecticidal specificity similar to schradan, namely the methosulfate of the thiol isomer of demeton (XXV) (68b) and the *O,O*-diethyl S-2-diethylaminoethyl phosphorothiolate (XXVI) (77).



$$(EtO)_2\overset{\overset{\displaystyle O}{\|}}{P}SCH_2CH_2\underset{\underset{\displaystyle [CH_3SO_4]^-}{+}}{\overset{\overset{\displaystyle CH_3}{|}}{S}}CH_2Me$$

(XXV)



$$(EtO)_2\overset{\overset{\displaystyle O}{\|}}{P}SCH_2CH_2NEt_2$$

(XXVI)

A comparative toxicological assessment of these compounds is still lacking, but from the data on insect toxicity given by Fukuto *et al.* (68b) and Gosh and Newman (77) a similarity to the selective toxicity of schradan is obvious. The compounds (XXV) and (XXVI) are almost totally ionized at physiological pH's; Spencer and O'Brien suggested that the introduction of an ionizable group produces selective toxicity whereby aphids, Tetranychid mites and mosquito larvae were susceptible insects; the mammalian toxicity is not reduced (207). Several other systemics show some selectivity. Isolan is toxic to aphids and ineffective against red spider mites, and such natural enemies as Anthocorids and Chalcids, but is toxic to the larvae of *Coccinellidae*, *Syrphidae* and *Chrysopidae* (81).

Several of the most useful systemics combine contact toxicity, vapour toxicity (33,43,47,241) with systemic effects and are therefore not selective if applied as foliage sprays. Demeton or methyl demeton are very toxic to Syrphid larvae, certain Coccinellids, and to Hymenopterous parasites. Mathys found demeton equally toxic to the fruit tree red spider, *Metatetranychus ulmi* (Koch) and the predacious mite, Typhlodromus sp. (141,142) as is methyl demeton (143).

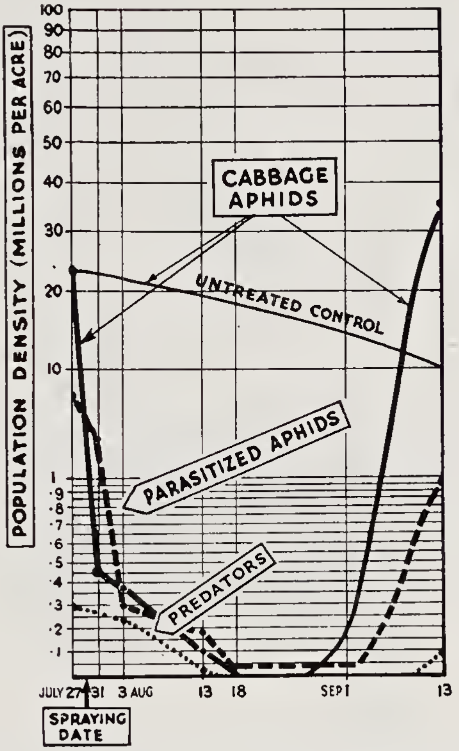


Fig. 2. Population of cabbage aphis and its natural enemies after application of a selective systemic, schradan ($3^{1}/_{2}$ pt. of Pestox 3® per acre) (Ripper (178)).

Certain nonselective systemics can, however, be used to achieve ecological selectivity in the form of seed treatment, soil application, bark treatment, or trunk implantation if the insecticide is unavailable to the natural enemies. This phenomenon was first demonstrated for dimefox and mipafox (182) and has since been confirmed in many cases (150), but recently a case was reported where demeton applied as soil treatment against a leafhopper affected its egg predator, a Mirid *Cyrtorrhinus mundulus* (Breddin) for a few days after soil application (225).

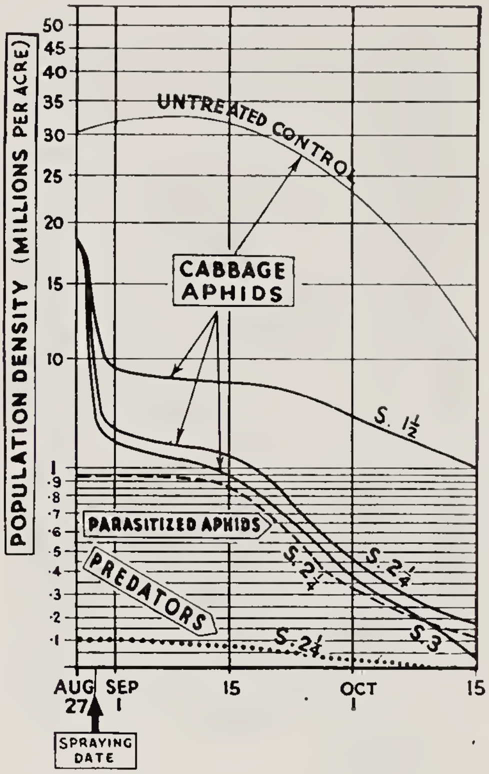


Fig. 3. Populations of cabbage aphis and its natural enemies after treatment with 3 reduced dosages of schradan (S.3 indicates 3 pt. per acre, S.$2^{1}/_{4}$ $2^{1}/_{4}$ pt., S.$1^{1}/_{2}$ $1^{1}/_{2}$ pt. of Pestox 3®). The populations of the predators and the parasitised aphids are inserted for the medium dosage only (Ripper (178)).

Selective systemics have been used successfully without resurgences of pests which do resurge after treatment with nonselective ectotherapeutical insecticides or nonselective systemics (177,178). Resurgence often arises from a multiplication of survivors which is rapid, because the unselective insecticide has removed the check of the biotic resistance of the ecosystem. The natural enemies of the pest survive after a treatment by a selective insecticide, and attack any individuals of the pest which survive the treatment. The high density dependent natural enemies leave first when the pest population has fallen to a level no longer attractive to them, and the low density dependent natural enemies are left to carry on until the crop is completely cleared of pests. Thus a selective insecticide if applied in the recommended dosage ensures a clean crop; this is, however, in due course reinfested by immigrant individuals of the pest species, and a new infestation builds up from them (see Fig. 2) (179). This bears out the conception by Boyce, Nicholson, and Wigglesworth who have forecast that the reduction of the phytophagous species to a low population density by the application of pesticides would produce an increase of the biotic potential of the pest, arising out of the simultaneous reduction of the biological control factors whether they are due directly to the insecticide killing the natural enemies or indirectly by

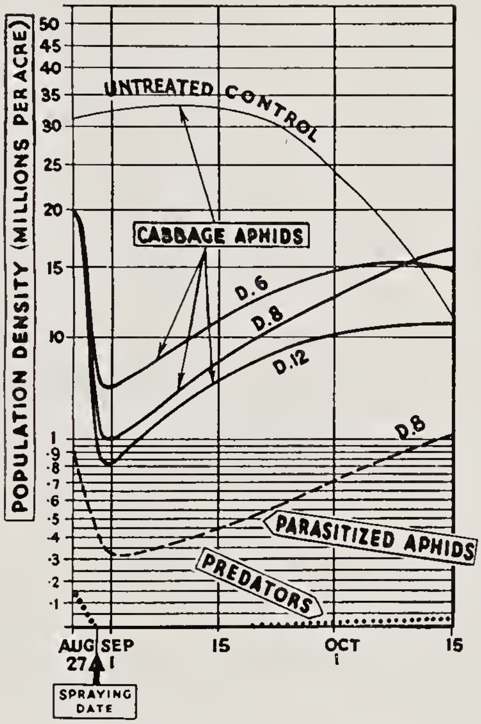


Fig. 4. Populations of cabbage aphis and its natural enemies after treatment with the non-selective systemic demeton at the 3 reduced concentrations; (D.12 indicates 12 oz., D.8 8 oz., and D.6 6 oz. of Systox® (Ripper (178)).

being starved out (179). It follows therefore that selective insecticides should be so used as to offset only the effect of the factors which have upset the balance of population and led to the pest outbreak without so reducing the pest population that the natural enemies can eliminate them completely and then disappear themselves. A reduction of the population density of the pest seemed desirable only to a level which does not produce damage to the crop but is sufficiently high to maintain the low density natural enemies. I therefore

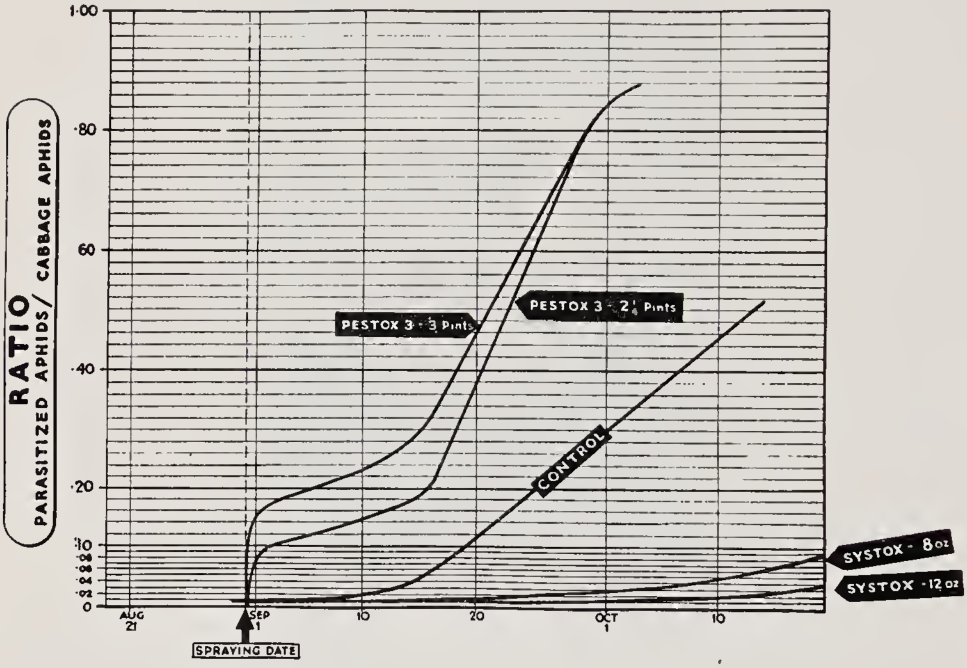


Fig. 5. The ratio of the population of parasitised aphids and cabbage aphis during an untreated aphis outbreak, after treatments with a selective insecticide systemic, schradan, as Pestox 3®, and non-selective systemic insecticide, demeton, as Systox® (Ripper (178)).

carried out experiments with the cabbage aphid on brassica crops, in which dosages of the selective systemic insecticide schradan were so reduced that they no longer led to a complete control of the aphis but established a new equilibrium between the pest and the low density natural enemies at a lower level of the population (Fig. 3); similar applications of reduced quantities of unselective systemics, namely demeton and methyl demeton, led to severe resurgences (Fig. 4) (178). After the treatment with the reduced quantities of schradan the plots showed a small aphis population which slowly declined toward the end of the season. At harvest time the crop was free of the pest in spite of the fact that migrants of the cabbage aphis appeared right through this period; on the plots treated with nonselective systemics the crops were spoiled by resurgences. The explanation of the difference in the behavior of aphid populations after the selective insecticides and nonselective systemics appeared in a study of the population dynamics. On the plots treated with the nonselective systemic insecticides a negative correlation existed between the individuals of the resurging pest and the number of natural enemies, while in the case of the selective insecticide, the ratio of natural enemies to pests was very much increased (Fig. 5) (178).

Similar differences in the reaction to treatments with schradan and demeton and the marked superiority of the former against aphids have been observed by Michelbacher *et al.* in California where a single schradan treatment against the walnut aphid (155a and b) resulted in excellent aphid suppression for a full year.

An alteration of the balance between pests and natural enemies in such a way as is conducive to maintain sufficient pest population after treatment as will retain the low density dependent natural enemies on the crop is an example of successful integration of biological and chemical control. To achieve a repetition of such integration in the more complex pest problems with which we are often confronted requires the discovery of many more selective insecticides. On the other hand, the present selective systemics suffice to explore this phenomenon further. This is now being done by the use of variable dosage sprayers (178), which is a convenient instrument to study the effect of pesticides on the balance of arthropod populations in the treated ecosystem. In order to progress the integration of biological and chemical control more knowledge of the ecology of low density dependent natural enemies is required; in particular it is necessary to know the minimum population density of the host at which the natural enemies maintain themselves and retain interest in an infested crop and further the ratio between natural enemies and pest at which the natural enemies can keep the pest population in a semiequilibrium. In regard to ecology of the pest, a knowledge of the maximum population density which does not cause significant damage to the crop is important.

A study of the *known* undesirable effects of pesticides, i.e., resurgences, development of man-made pests and the segregation of resistant strains, make it seem likely that many of these sequels can be avoided by combination of biological and chemical control methods (179). In view of the menace of resistance bred from genotypic variants who survive pesticidal treatment, the elimination of such resistant survivors by natural enemies gives crop protection a preventive measure which medical entomology unfortunately does not possess and one which, in the opinion of the writer, we cannot afford to leave unexploited. A great volume of knowledge on biological control has so far remained unused, because the colonization of natural enemies has chiefly been directed toward biological control by itself. It seems probable that there are many natural enemies particularly low density dependent species available which would supplement the control effected by selective insecticides.

The success of selective systemics calls for a reappraisal of systemic compounds which all too often have been judged solely by the percentage of mortality achieved (145) without consideration whether a near 100% kill was desirable and whether the natural enemies of the pest were also affected. The rapid increase of the number of pests which have acquired resistance to insecticides justifies, in the writer's opinion, the use of selective insecticides even at a higher price than unselective pesticides in order to preserve the natural biotic resistance or to allow the introduction of low density dependent natural enemies if such do not occur in the ecosystem. This is particularly important where a large percentage of crops are sprayed, for example, in the case of prophylactic sprays used in horticulture or against aphis to prevent the spread of viruses, as such repeated spraying reduces the populations of natural enemies over wide areas. For the same reason and in view of the existing knowledge of the physiology of resistance (106, 149), a much greater effort to find further selective systemic insecticides is warranted. If further experience should show that the development of resistance is a general phenomenon of which many phytophagous pests are capable, developing sooner or later against every repeatedly applied insecticide, and that resistance against one insecticide accelerates the development of resistance against other compounds, then it would indeed be urgent to find selective insecticides and to introduce them in crop protection before resistance against other insecticides makes their use too difficult by stultifying their initial control effect.

One prospect to obtain new physiological selective insecticides lies in the further study of zoometatoxicity. In an analogous way new selective herbicidal compounds were synthesized by Wain (226) through adding to herbicidal molecules side chains which only certain plants β-oxidize to the herbicidal rest of the molecule.

It must also not be overlooked that systemic insecticides after good initial control pass much quicker through the phase in which they could "select" resistant individuals in a pest population than ectotherapeutical insecticides and are more quickly destroyed by soil organisms than many of the widely used insecticides for which the segregation of resistant strains has already been reported.

## References

1. Ahmed, M. K., *J. Econ. Entomol.*, *48*, 530–2 (1955).
2. Ahmed, M. K., Newsom, L. D., Roussel, J. S., and Emerson, R. B., *J. Econ. Entomol.*, *47*, 684–91 (1954).
3. Anderson, R. F., *J. Econ. Entomol.*, *48*, 187–90 (1955).
4. Anon., *Hanane, A Systemic Insecticide*, Pest Control Ltd., 1954.
5. Anonymous, *Thimet Insecticide Technical Manual*, American Cyanamid Co., 1956.
6. Apple, J. W., and Martin, R., *J. Econ. Entomol.*, *48*, 193–5 (1955).
7. Augustinsson, K. B., and Heimburger, G., *Acta Chem. Scand.*, *8*, 753–61 (1954).
8. Barthel, W. F., Alexander, B. H., Giang, P. A., and Hall, S. A., *J. Am. Chem. Soc.*, *77*, 2424–7 (1955).
9. Bennett, S. H., *Ann. Rev. Entomol.*, *2*, 279–296 (1957).
10. Bennett, S. H., and Thomas, W. D. E., *Proc. Isotope Techniques Conference*, Oxford, 1951.
11. Bennett, S. H., and Thomas, W. D. E., *Trans. 9th Intern. Congr. Entomol.*, *1*, 981 (1952).
12. Bennett, S. H., and Thomas, W. D. E., *Ann. Appl. Biol.*, *41*, 484–500 (1954).
13. Bergeson, G. B., *Plant Disease Reptr.*, *39*, 705–09 (1955).
14. Böhm, H., *Der Pflanzenarzt*, *8*, 87–8 (1955).
15. Böhm, H., *Der Pflanzenarzt*, *8*, 109 (1955).
16. Böhm, H., *Der Pflanzenarzt*, *9*, 15 (1956).
17. Böhm, O., *Der Pflanzenarzt*, *5*, 4–5 (1952).
18. Böhm, O., *Pflanzenschutz-Berichte*, *11*, 41–53 (1954).
19. Bond, J. A. B., *Bull. Entomol. Research*, *44*, 97 (1953).
20. Bovey, P., and Savary, A., *Rev. Romande Agric. Vitic.*, *5*, 1 (1949).
21. Broadbent, L., Burt, R. E., and Heathcote, G. D., *Ann. Appl. Biol.*, *44*, 256–273 (1956).
22. Cangardel, H., private communication, 1956.
23. Carmen, G. E., and Metcalf, R. L., *Citrus Leaves*, October 1951.
24. Casida, J. E., *Science*, *122*, 597–8 (1955).

25. Casida, J. E., *Biochem. J.*, *60*, 487–96 (1955).
26. Casida, J. E., Allen, T. C., and Stahmann, M. A., *Nature*, *172*, 243 (1955).
27. Casida, J. E., Chapman, R. K., Stahmann, M. A., and Allen, T. C., *J. Econ. Entomol.*, *47*, 64–70 (1954).
28. Casida, J. E., Getzin, L. W., Jr., and Chapman, R. K., *J. Agr. Food Chem.*, *4*, 236 (1956).
29. Caughey, W., and Bond, J. A. B., *British Patent Applic.* 5453–55 (1955).
30. Champ, B. R., *Queensland Agr. J.*, October 1954.
31. Clark, E. L., Johnson, G. A., and Mattson, E. L., *J. Agr. Food Chem.*, *3*, 834–6 (1955).
32. Corey, R. A., Dorman, S. C., Hall, W. E., and Glover, L. C., *J. Econ. Entomol.*, *46*, 386–7 (1953).
33. Corey, R. A., Dorman, S. C., Hall, W. E., Glover, L. C., and Whetstone, R. R., *Science*, *118*, 28–9 (1953).
34. Crowdy, S. H., Grove, J. F., Hemming, H. G., and Robinson, K. C., *J. Exptl. Bot.*, *7*, 42–64 (1956).
35. Dame, F., *Landw. Wochenblatt Westfalen*, *112*, 179 (1955).
36. Dame, F., and Goossen, H., *Höfchen-Briefe*, *7*, 78 (1954).
37. Davich, T. B., and Apple, J. W., *J. Econ. Entomol.*, *44*, 528–33(1951).
38. Davich, T. B., and Apple, J. W., *J. Econ. Entomol.*, *48*, 180–1 (1955).
39. David, W. A. L., *Ann. Appl. Biol.*, *38*, 508–24 (1951).
40. David, W. A. L., and Gardiner, B. O. C., *Ann. Appl. Biol.*, *39*, 203–10 (1952).
41. David, W. A. L., and Gardiner, B. O. C., *Ann. Appl. Biol.*, *40*, 91–105 (1953).
42. David, W. A. L., and Gardiner, B. O. C., *Ann. Appl. Biol.*, *40*, 403 (1953).
43. David, W. A. L., and Gardiner, B. O. C., *Bull. Entomol. Research*, *45*, 683–92 (1954).
44. David, W. A. L., and Gardiner, B. O. C., *Bull. Entomol. Research*, *45*, 693–702 (1954).
45. David, W. A. L., and Gardiner, B. O. C., *Ann. Appl. Biol.*, *41*, 261 (1954).
46. David, W. A. L., and Gardiner, B. O. C., *Ann. Appl. Biol.*, *43*, 594–614 (1955).
47. Davis, D. W., and Sessions, A. C., *J. Econ. Entomol.*, *46*, 526–7 (1953).
48. Davson, H., and Danielli, J. F., *The Permeability of Natural Membrane*, Cambridge University Press, London, 1952, 255 pp.
49. De Pietri-Tonelli, P., and March, R. B., *J. Econ. Entomol.*, *47*, 902–8 (1954).
50. De Pietri-Tonelli, P., *Agr. Ital. (Rome)*, *93*, 49–56 (1956).
51. De Pietri-Tonelli, P., *Nuovi Mezzi di lotta contro la Rhagoletis cerasi L con l'impiego di N-dialchilammidi di acidi o,o-dialchiltiofosforilacetici*, Montecatini, 1956 30 pp.
52. De, R. K., and Konar, G., *J. Econ. Entomcl.*, *49*, 250–1 (1956).
53. Dowdy, A. C., and Slessman, J. P., *J. Econ. Entomol.*, *45*, 640–3 (1952).
54. Downing, R. S., *Proc. Entomol. Soc. Brit. Columbia*, *49*, 177 (1953).
55. Du Bois, K. P., and Coon, J. M., *Arch. Ind. Hyg. Occupational Med.*, *6*, 9–13 (1952).
56. Dunning, R. A., *British Sugar Beet Review*, *24*, 121 (1956).

57. Dupee, L. F., Heath, D. F., and Otter, I. K. H., *J. Agr. Food Chem.*, *4*, 233–6 (1956).

58. Duspiva, F., *Mitt. Biol. Zentralanst. Land-Forstwirtsch. Berlin-Dahlam*, *70*, 91 (1951).

59. Edgar, E. C., *Outlook on Agriculture*, *1*, 16–19 (1956).

60. Edson, E. F., *Agricultural Mercht.*, February 1956.

61. Ehrenhardt, H., *Anz. Schädlingskunde*, *27*, 1 (1954).

62. Emery, G. A., Paper given at *Natl. Crop Protection Conf.*, Eastbourne, England, 1955.

63. Ernould, L., *Publications Techniques de l'I. B. A. B.*, *3*, 147 (1955).

64. Feder, W. A., *N. Y. State Flower Growers, Bull.*, *No. 82* (1952).

65. Frohberger, E., *Höfchen-Briefe*, *2*, 10 (1949).

66. Fuchs, W. H., and Kunz, H. D., *Naturwiss.*, *41*, 20 (1954).

67. Fukuda, J., Okudai, S., Shinkaji, N., Koremura, M., *Hort. Div., National Tokai-Kinki Agric. Exp. Sta., Japan, Special Rept.*, *3*, 39–69 (1956).

68a. Fukuto, T. R., Metcalf, R. L., March, R. B., and Maxon, M. G., *J. Econ. Entomol.*, *48*, 347–54 (1955).

68b. Fukuto, T. R., Metcalf, R. L., March, R. B., and Maxon, Marion, *J. Am. Chem. Soc.*, *77*, 3670 (1955).

69. Fulton, R. A., and Mason, H. C., *Science*, *85*, 2202 (1937).

70. Gasser, R., *Ber. schweiz. botan. Ges.*, *62*, 66 (1952).

71. Gasser, R., *III^e^ Congr. intern. phytopharmacie, Paris, France*, 1952.

72. Gasser, R., *Z. Naturforsch.*, *8b*, 225 (1953).

73. Gast, A., *C. R. Congr. Pomologique de France, Alger*, *83*, 177 (1952).

74. Giang, P. A., *USDA Agr. Research Servicc, Ent. Res. Branch*, *E-874* (1954).

75. Glass, E. H., and Chapman, P. J., *J. Econ. Entomol.*, *48*, 695–7 (1955).

76. Goetz, B., private communication, 1956.

77. Gosh, R., and Newman, J. F., *Chemistry & Industry*, *1955*, 118.

78. Grandori, R., and Rota, P. A., *Boll. Zool. Agr. Bachic.*, *21*, 53–68 (1955).

79. Greenslade, R. M., *Proc. 2nd Intern. Congr. Crop Protection*, *1949*, 158–61 (1951).

80. Greenslade, R. M., *Chemistry & Industry*, *1953*, 1223–4.

81. Grob, H., *III^e^ Congres international phytopharmacie, Paris, France*, 1952.

82. Günthart, E., *Mitt. schweiz. entomol. Ges.*, *18*, 648 (1945).

83. Haines, R. G., in press.

84. Hanna, A. D.. Judenko, E., and Heatherington, W., *Ann. Appl. Biol.*, *45*, in press (1957).

85. Hanna, A. D., Judenko, E., and Heatherington, W., *Bull. Entomol. Research*, *46*, 669–710 (1955).

86. Hanna, A. D., and Nicol, J., *Nature*, *173*, 730 (1954).

87. Hartley, G. S., *XIIth Intern. Congr. Pure & Applied Chemistry*, New York, 1951.

88. Hartley, G. S., *World Crops*, *4*, 397 (1952).

89. Hartley, G. S., *III^e^ Congres international phytopharmacie, Paris, France*, 1952.

90. Hartley, G. S., *Chemistry & Industry*, *1954*, 529–32.

91. Hartley, G. S., and Heath, D. F., *Nature*, *167*, 316 (1951).

92. Hayes, W. J., and Pearce, G. W., *Agr. Food Chem.*, *1*, No. 6, 966 (1953).
93. Hayes, W. J., *Publ. Health Repts.*, *69*, No. 10, 893 (1954).
94. Heath, D. F., Cleugh, J., Otter, I. K. H., and Park, P. O., *J. Agr. Food Chem.*, *4*, 230–3 (1956).
95. Heath, D. F., Lane, D. W. J., and Llewellyn, M., *J. Sci. Food Agr.*, *2*, 60 (1952).
96. Heath, D. F., Lane, D. W. J., and Park, P. O., *Trans. Roy. Soc. (London)*, *B239*, 191–214 (1955).
97. Heath, D. F., Park, P. O., Lickerish, L. A., and Edson. E. F., Pest Control Ltd. (Mimeo Report, 1953.)
98. Heinze, K., *Mitt. Biol. Bundesanstalt Land-Forstwirtschaft Berlin-Dahlem*, *80*, 81 (1954).
99. Henderson, C. F., and Tilton, E. W., *J. Econ. Entomol.*, *48*, 157–61 (1955).
100. Hille Ris Lambers, D., Reestman, A. J., and Schepers, A., *Ned. J. Agric. Sci.*, *1*, 188–201 (1953).
101. Hills, O. A., private communication, 1956.
102. Hirschmann, J., *Höfchen-Briefe*, *6*, 90–113 (1953).
103. Hofferbert, W., and Orth, H., *Höfchen-Briefe*, *5*, 10–15 (1952).
104. Hoof van, H. A., *Tijdschr. Plantenziekten*, *60*, 131–5 (1954).
105. Horiuchi, N., *Takamine Ann. Research Rep.*, *7*, 209–214 (1956).
106. Hoskins, W. M., and Gordon, H. T., *Ann. Rev. Entomol.*, *1*, 89–122 (1956).
107. Hull, R., *Agriculture*, *61*, 205–10 (1954).
108. Hull, R., *Crop Protection Conference*, Eastbourne, England, 1955.
109. Hull, R., and Gates, L. F., *Ann. Appl. Biol.*, *40*, 60–78 (1953).
110. Hutchinson, M. T., *J. Econ. Entomol.*, *48*, 222–3 (1955).
111. Ivy, E. E., *Agr. Chemicals*, *7*, 44–45, 121–123 (1952).
112. Ivy, E. E., *Agr. Chemicals*, *8*, 47 (1953).
113. Ivy, E. E., Scales, A. L., and Gorzycki, L. J., *J. Econ. Entomol.*, *47*, 1148–9 (1954).
114. Iyatomi, K., *Plant Protection*, *9*, 355–8 (1955).
115. Jacks, H., *New Zealand J. Sci. Technol.*, *A 36*, 454–9 (1955).
116. Jacks, H., private communication, 1956.
117. Jary, S. G., private communication, 1956.
118. Jeppson, L. R., *J. Agr. and Food Chem.*, *1*, 830–2 (1953).
119. Jeppson, L. R., Jesser, M. J., and Complin, J. O., *J. Econ. Entomol.*, *45*, 669–71 (1952).
120. Goosen, H., *Landw. Wochenblatt Westfalen*, *112*, 5–6 (1955).
121. Kantack, E. J., *J. Econ. Entomol.*, *48*, 486 (1955).
122. Kawamura, T., and Nomura, K., *Agriculture & Horticulture*, *29*, 1421–4 (1954).
123. Kawashire, I., Fujii, S., Hayashi, T., Ejima, A., and Kato, S., *Bull. Nat. Hyg. Lab., Tokyo, No. 73*, 205–11 (1955).
124. Kilby, B. A., *Chemistry & Industry*, *1953*, 856–61.
125. Klostermeyer, E. C., Landis, B. J., Schoop, R., and Butler, L. I., *J. Econ. Entomol.*, *49*, 164–166 (1956).
126. Kocher, C., Roth, W., and Treboux, J., *Anz. Schädlingskunde*, *26*, 65 (1953).
127. Koppelberg, B., and Steudel, W., *Zucker*, *No. 7*, 1956.

128. Kunz, H. D., *Z. Pflanzenkrankh. u. Pflanzenschutz*, *61*, 481–520 (1954).
129. Lanchester, H. P., *J. Econ. Entomol.*, *47*, 1020–1 (1954).
130. Lane, D. W. J., and Heath, D. F., *British Patent Specification* 742,796 (1952).
131. Langenbuch, R., *Nachrbl. deut. Pflanzenschutzdienst*, *3*, 118 (1951).
132. Lekander, B., *Trans. IXth Intern. Congr. Entomol.*, 679–81 (1952).
133. Lilly, J. H., and Fahey, J. E., *J. Econ. Entomol.*, *49*, 815–8 (1956).
134. Lochner, E. H. W., *Union S. Africa, Dept. Agr. Sci. Bull., No. 333* (1952).
135. Lüdicke, M., *Anz. f. Schädlingskunde*, *22*, 58 (1949).
136. Lüdicke, M. Z., *Pflanzenkrank. u. Pflanzenschutz*, *56*, 31 (1949).
137. March, R. B., Fukuto, T. R., and Metcalf, R. L., *Agr. Chemicals*, *9*, 33, 107, 128–30 (1954).
138. March, R. B., Metcalf, R. L., and Fukuto, T. R., *J. Agr. Food Chem.*, *2*, 732–5 (1954).
139. March, R. B., Metcalf, R. L., Fukuto, T. R., and Maxon, M. G., *J. Econ. Entomol.*, *48*, 355–63 (1955).
140. Martin, H., and Shaw, H., *BIOS Final Report* 1095, 1946.
141. Mathys, G., *Rev. romande agr., viticult. et aboricult.*, *10*, 81–4 (1954).
142. Mathys, G., *Annuaire agr. de la Suisse*, *56*, 815–25 (1955).
143. Mathys, G., *Rev. romande agr. viticult. et aboricult.*, *12*, 3–5 (1956).
144. Mazur, A., *J. Biol. Chem.*,*164*, 271–89 (1946).
145. McLeod, W. S., *Canada Dept. Agr., Science Service*, *E-7* (1952).
146. Medler, J. T., *J. Econ. Entomol.*, *48*, 718–23 (1955).
147. Metcalf, R. L., private communication, 1956.
148. Metcalf, R. L., *Organic Insecticides*, Interscience Publishers, Inc., New York, 1955.
149. Metcalf, R. L., *Physiol. Revs.*, *35*, 197–232 (1955).
150. Metcalf, R. L., *Second International Plant Protection Conference, Fernhurst*, *23*, 1956.
151. Metcalf, R. L., and March, R. B., *Ann. Entomol. Soc. Amer.*, *46*, 63 (1953).
152. Metcalf, R. L., March, R. B., Fukuto, T. R., and Maxon, M., *J. Econ. Entomol.*, *47*, 1045–55 (1954).
153. Metcalf, R. L., March, R. B., Fukuto, T. R., and Maxon, M., *J. Econ. Entomol.*, *48*, 364–9 (1955).
154. Metcalf, R. L., Fukuto, T. R., March, R. B., and Stafford, E. M., *J. Econ. Entomol.*, *49*, 738–741 (1956).
155a. Michelbacher, A. E., *J. Econ. Entomol.*, *48*, 504–9 (1955).
155b. Michelbacher, A. E., and Oatman, E., *J. Econ. Entomol.*, *48*, 768–9 (1955).
156. Morrison, H. E., and Thompson, B. G., *J. Econ. Entomol.*, *48*, 706–10 (1955).
157. Mühlmann R., and Tietz, H., *Höfchen-Briefe*, *9*, 116–140 (1956).
158. Nicol, J., *Nature*, *169*, 120 (1952).
159. Nicol, J., *Nature*, *173*, 36 (1954).
160. Nomura, H., private communication, 1956.
161. Nomura, K., *Technical Bull. Faculty of Horticulture, Chiba University, Japan*, *2*, 25 (1954).
162. Nomura, K., *Agriculture & Horticulture*, *30*, 235–8 (1955).
163. Nomura, K., *Plant Protection* (*Japan*), *9*, 314–16 (1955).

164a. O'Brien, R. D., and Spencer, E. Y., *J. Agr. Food Chem.*, *1*, 946–50 (1953).

164b. O'Brien, R. D., and Spencer, E. Y., *J. Agr. Food Chem.*, *3*, 56 (1955).

165. Okinaka, A. J., Doull, J., Coon, J. M., and DuBois, K. P., *J. Pharmacol. Exptl. Therap.*, *112*, 231–45 (1954).

166. Otter, I. K. H., *Mikrochim. Acta*, *1*, 125–33 (1956).

167. Park, P. O., and Heath, D. F., *IIIᵉ Congres international phytopharmacie, Paris, France*, 1952.

168. Pellegrind, G., *Agr. ital.* (*Rome*), *92*, 56–57 (1955).

169. Perucci, E., *Tabacco*, *58*, 279–304 (1954).

170. Petty, B. K., and Lochner, E. H. W., *Union S. Africa, Dept. Agr. Sci. Bull., No. 343* (1953).

171. Proverbs, M. D., *Contribution 3174, Entomol. Div., Science Service, Dept. of Agric., Ottawa*, 1954.

172. Questel, D. D., and Connin, R. V., *J. Econ. Entomol.*, *40*, 914 (1947).

173. Rainwater, C. F., *P. M. Release*, December 16, 1955.

174. Reynolds, H. T., *Agr. Chemicals*, *9*, 28–31, 113, 115 (1954).

175. Reynolds, H. T., private communication, 1956.

176. Reynolds, H. T., and Anderson, L. D., *J. Econ. Entomol.*, *48*, 671–5 (1955).

177. Ripper, W. E., *3rd Intern. Congr. Crop Protection*, Paris, France, 1952.

178. Ripper, W. E., *Proc. 10th Intern. Congr. Entomol.*, Montreal, 1956.

179. Ripper, W. E., *Ann. Rev. Entomol.*, *1*, 403–38 (1956).

180. Ripper, W. E., *Brit. Patent applic.* 1895/56 (1956).

181. Ripper, W. E., Greenslade, R. M., and Hartley, G. S., *Bull. Entomol. Research*, *40*, 481–502 (1950).

182. Ripper, W. E., Greenslade, R. M., and Hartley, G. S., *J. Econ. Entomol.*, *44*, 448–59 (1951).

183. Ripper, W. E., *Bull. Entomol. Research* in prepn., 1957.

184. Roland, G., *Parasitica*, *9*, 125–31 (1953).

185. Rönnebeck, W., *Nachrbl. Deu. Pflanzenschutzdienst*, *4*, 189 (1952).

186. Rönnebeck, W., *Z. Pflanzenkrankh. u. Pflanzenschutz*, *59*, 13–26 (1952).

187. Rümker von, R., *Agr. Chemicals*, *10*, 47–48, 122–123 (1955).

188. Rümker von, R., *Agr. Chemicals*, *10*, 41–43, 131–133 (1955).

189. Rümker von, R., *Agr. Chemicals*, *10*, 40–42, 103–105 (1955).

190. Saggers, D. T., *Fisons Pest Control Ltd., Report*, 1956.

191. Salzmann, R., Schmidhauser, P., and Meier, W., *Mitt. schweiz. Landwirtschaft*, *1*, 97 (1953).

192. Sasser, J. N., *Plant Disease Reptr.*, *36*, 228 (1952).

193. Schneider, F., *Schweiz. Z. Obst- u. Weinbau., Obstrundschau*, *58*, 33 (1949).

194. Schrader, G., *Höfchen-Briefe*, *5*, 161 (1952).

195. Schrader, G., *Monographien zu "Angewandte Chemie" und "Chemie-Ingenieur-Technik*, *62* (1952).

196. Schreier, O., and Russ, K., *Pflanzenschutz-Berichte*, *13*, 1–43 (1954).

197. Schuder, D. L., *Natl. Shade Tree Conf. Proc.*, *29*, 108–12 (1953).

198. Schuhmann, G., *Höfchen-Briefe*, *6*, 233–82 (1953).

199. Seki, M., Hirokawa, N., and Ishida, T., *Citrus Division, Saga Agric. Exptl. Sta., Japan, Report 1*, 1–28 (1954).

200. Sekitani, I., Hayakawa, H., Kurobane, K., Yanagi, T., and Yamagishi, X., *Ministry of Education Scientific Experiment Research Report, Nagano Prefectual Agric. Exptl. Sta.*, Japan (1955).
201. Selke, K., *Nachrbl. deut. Pflanzenschutzdienst*, *4*, 221 (1950).
202. Sher, S. A., Kamasaki, H., and Murakishi, H., *Hawaii Farm Science*, July (1953).
203. Sherrer, J. D., and Arant, F. S., *J. Econ. Entomol.*, *46*, 1116–17 (1953).
204. Simon, F. (J. E.), *Inf. Estac. Exp. Agric. La Molina*, *90* (1954).
205. Smith, F. F., and Fulton, R. A., *J. Econ. Entomol.*, *43*, 627–32 (1950).
206. Spencer, E. Y., and O'Brien, R. D., *J. Agr. Food Chem.*, *1*, 716–20 (1953).
207. Spencer, E. Y., and O'Brien, R. D., *Ann. Rev. Entomol.*, *2*, 261–278 (1957).
208. Spindler, M., *Z. Pflanzenkrankh. u. Pflanzenschutz*, *62*, 98–165 (1955).
209. Steudel, W., and Heiling, A., *Mitteilungen aus der Biologischen Zentralanstalt f. Land- u. Forstwirtschaft Berlin-Dahlem*, *79* (1954).
210. Steudel, W., and Heiling, A., *Nachrbl. deut. Pflanzenschutzdienst*, *8*, 1 (1956).
211. Steudel, W., and Heiling, A., *Zucker*, *8*, 207–12 (1955).
212. Swenson, K. G., Davis, A. C., and Schroeder, W. T., *J. Econ. Entomol.*, *47*, 490 (1954).
213. Takeuchi, T., Horiuchi, N., and Kuwada, G., *5th Ann. Rept. Takamine Laboratory*, Sankyo Co. Ltd., Japan, 132–43 (1953).
214. Tanaka, M., private communication, 1956.
215. Taschenberg, E. F., and Avens, A. W., *J. Econ. Entomol.*, *48*, 685–8 (1955).
216. Thomas, W. D. E., and Bennett, S. H., *Ann. Appl. Biol.*, *41*, 501–19 (1954).
217. Thomas, W. D. E., Bennett, S. H., and Lloyd-Jones, C. P., *Ann. Appl. Biol.*, *43*, 569–93 (1955).
218. Tietz, H., *Farbenfabriken Bayer* (1954).
219. Tietz, H., *Höfchen-Briefe*, *7*, 1–55 (1954).
220. Tsuyuki, H., Stahmann, M. A., and Casida, J. E., *J. Agr. Food Chem.*, *3*, 922–32 (1955).
221. Unterstenhöfer, G., *Z. Pflanzenkrankh. u. Pflanzenschutz*, *57*, 272–81 (1950).
222. Unterstenhöfer, G., *Gesunde Pflanzen*, *4*, 257–260 (1952).
223. Unterstenhöfer, G., *Höfchen-Briefe*, *6*, 121–30 (1953).
224. Upholt, W. M., *Agr. Food Chem.*, *3*, No. 12, 1000 (1955).
225. Verma, J. S., *J. Econ. Entomol.*, *49*, 58–63 (1956).
226. Wain, R. L., *J. Agr. Food Chem.*, *3*, 128–30 (1955).
227. Walrave, J., *Tijdschr. Plantenziekten*, *60*, 93–108 (1954).
228. Watson, M. A., *Proc. Roy. Soc. (London)*, *B133*, 200–19 (1944).
229. Watson, M. A., and Roberts, F. M., *Proc. Roy. Soc. (London)*, *B127*, 543–76 (1939).
230. Wayne, A. B., and Arant, F. S., *J. Econ. Entomol.*, *47*, 1111–14 (1954).
231. Wedding, R. T., *J. Agr. Food Chem.*, *1*, 832–4 (1953).
232. Wedding, R. T., and Metcalf, R. I., *Botan. Gaz.*, *114*, 180 (1952).
233. Wenzl, H., and Krexner, R., *Pflanzenschutzberichte*, *12*, 105–28 (1954).
234. Wiesmann, R., Gasser, R., and Grob, H., *Experientia*, *7*, 117 (1951).
235. Wilcox, J., and Howland, A. F., *J. Econ. Entomol.*, *48*, 581–3 (1955).
236. Wilcox, J., and Howland, A. F., *J. Econ. Entomol.*, *48*, 579–81 (1955).

237. Wirth, W., *Arch. exptl. Pathol. Pharmakol.*, *217*, 144–52 (1953).
238. Wirth, W., *Deut. med. Wochschr.*, *79*, 1205–8 (1954).
239. Wollerman, E. H., *Proc. Ann. Meeting, Central Branch Entomol. Soc. Am.*, *10*, 28 (1955).
240. Zanardi, D., *Notiz. malattie piante (Italy), No. 34*, 51 (1956).
241. Zattler, F., *Höfchen-Briefe*, *4*, 131–69 (1951).

# CHEMICAL ANALYSIS OF PESTICIDE RESIDUES*

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## I. Introduction

Developments in physical and chemical (including enzymatic) methods of analysis of pesticide residues will be reviewed in this chapter. Organic pesticides will be emphasized because they are more numerous and seem to present more difficulties. Assay methods for bulk pesticides or formulations will be excluded since such methods are usually quite different from residue methods. Bioassay methods employing living organisms will be covered in the next chapter.

Problems relating to pesticide residues have greatly increased in importance during the last decade. There has been a great increase in the number of pesticides available, particularly organic pesticides, and in the volume in which they are used. Some pesticides, such as DDT, are stored in the body fat and have been shown to have cumulative toxic effects. Some, such as certain organophosphorus compounds, have a toxicity approaching that of the "nerve" war gases.

All those concerned with the proper use and application of pesticides—manufacturers, farmers, government officials, chemists, entomologists, pharmacologists, and the consuming public—agree on the desirability of making sure that there will be little or no hazard from the use of pesticides or from their residues. To this end, a number of important laws have been passed by many states and by the Federal government (435). Thus, the old Insecticide Act of 1910 was replaced with the Federal Insecticide, Fungicide and Rodenticide Act of 1947 requiring registration of pesticides used in interstate commerce and their proper labeling. The Federal Food, Drug and Cosmetic Act of 1938 contains Section 406 relating specifically to insecticidal residues. This act prohibits the unnecessary addition of poisons; where such additions are necessary, establishment of tolerances is authorized for insecticides and fungicides at such levels that the public health will be protected. Full implementation of this act was prevented by the intervention of World War II and the subsequent rapid development of many new pesticides. Hearings on tolerances were held in 1950 and finally the Miller amendment was passed in 1954, becoming public law 518. This law of outstanding importance prescribes the procedure for setting tolerances for pesticides on various crops.

Pesticide residues may be determined by analyses for some element present (chlorine, phosphorus, sulfur, arsenic, etc.), by enzymatic methods (cholinesterase inhibition), by bioassay, or by more specific methods. Nonspecific procedures should be used only where a specific method is not available, where they are faster and more economical and the time and expense of specific methods are not warranted, or where a knowledge of the residue problem has been obtained from experience with other methods. The problems faced in developing and applying these methods are challenging, particularly for organic compounds. The analyst is usually asked to determine microgram quantities of the pesticide in an extract containing large quantities of extraneous organic matter, which has to be removed to a degree depending on how much it interferes with the particular method of analysis used. The plant or animal extractive may contain related compounds formed during the manufacturing process for the technical pesticide as well as metabolites and degradation products of the applied pesticide. Some pesticides may be metabolized to even more toxic materials. Examples of this kind are the *in vivo* oxidation of schradan and demeton, and the conversion of heptachlor and of aldrin to their respective epoxides. In an analytical method, the pesticide, or an analytically determinable product therefrom, must be obtained in such a form that its determination will be relatively free from interferences from other materials in the plant or animal extract. These interferences should be eliminated or minimized even though blank runs are also used. The final quantitative determination can be made by any of the many spectrophotometric, electrometric, enzymatic, or other techniques available to the analyst.

The ultimate of desirability in a residue method is to be able to determine the amount of the residue as such and the amount of each of its metabolic or degradation products, for evaluation of the effectiveness of the pesticide or its of hazards. Such complete knowledge, if at all attainable, requires the use of all available techniques—physical, chemical, radiochemical, and bioassay—for isolation, identification, and analysis of products in a residue.

The validity of an analytical result depends not only on the accuracy of the final analysis, but also on the history of the sample. Poor sampling, inadequate handling and storage, or improper extraction techniques can negate the value of the most carefully performed analysis. Gunther (197) has discussed these factors fully. In this chapter we will assume that these preliminary steps have been satisfactorily accomplished and that the major problems are the cleanup procedures and the final analysis.

A number of recent books and reviews (17,197,308,313,317,339, 387,409,410) are available to those interested in the analysis of pesticide residues.

## II. Metabolism, Breakdown, Isomerization, and Oxidation of Pesticides

Residues may be lost by volatilization or by mechanical means. They also may be altered by moisture, light, and air, as well as by metabolism in plants, animals, or microorganisms. The products of such decomposition may be more or less toxic than the original pesticides, and furthermore it is desirable to know what their effects are on the analytical methods.

Even though the original pesticide is very toxic, the residue hazard may be negligible. Hazleton (218), in a review of the pharmacology of cholinesterase inhibitor insecticides, pointed out that under normal use conditions this seems to be true, since residues of many of these insecticides are negligible after several weeks.

The isomerization, metabolism, formation of breakdown products, and oxidation of pesticides have a direct bearing on residue methods. A few examples from those pesticides which have been studied in detail will be given.

### 1. DDT

DDT, a relatively stable insecticide, is metabolized in animals to DDE, which together with unchanged DDT is stored in fatty tissues (311,357); however, in the urine, the metabolic product is DDA (341,421).

### 2. Heptachlor

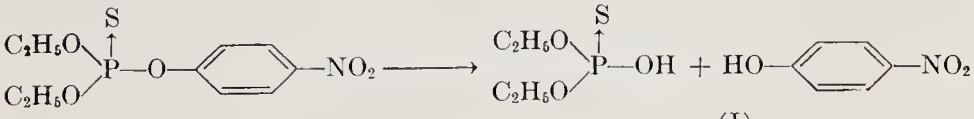
Davidow and Radomski (110) and Ely *et al.* (136) have shown that heptachlor epoxide is a metabolic product of heptachlor in animals. The epoxide is more toxic than heptachlor.

### 3. Aldrin

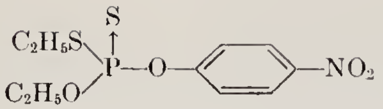
Aldrin is converted to the more toxic product, dieldrin, in carrots (181) and in animals (27).

### 4. Parathion

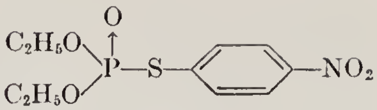
Parathion hydrolyzes slowly in water and more rapidly in alkali to *p*-nitrophenol and diethyl orthothiophosphoric acid (I) (264). Upon heating above 130° some *S*-ethyl isomer(II) is formed, and an *S*-nitrophenyl isomer (III) is also possible.



(I)



*S*-ethyl isomer
(II)



*S*-nitrophenyl isomer
(III)

Small amounts of para-oxon (diethyl *p*-nitrophenyl phosphate) and *p*-nitrophenol may be present. Methyl parathion, the *O,O*-dimethyl analogue of parathion, hydrolyzes and isomerizes more readily than parathion. The thermal isomerization of parathion, methyl parathion, malathion, and EPN has been studied by Metcalf and March (322).

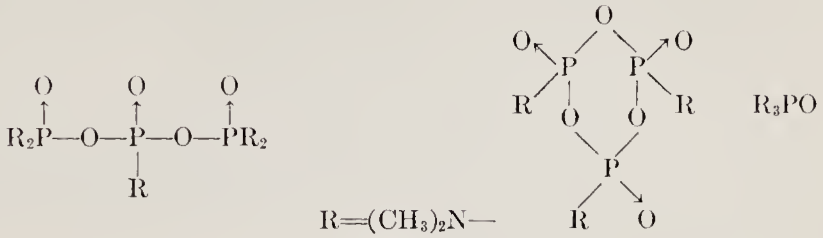
Parathion, methyl parathion, and probably other thiono and dithiophosphates are enzymatically metabolized to more active cholinesterase inhibitors by oxidation of the thiono sulfur atom to an oxygen atom (5,117,165,272,318); thus parathion is converted to para-oxon. Aldridge (3) has shown that mammalian A-esterase can hydrolyze para-oxon to *p*-nitrophenol and diethylphosphoric acid. Pure parathion is a very weak cholinesterase inhibitor, but the *S*-ethyl and *S*-nitrophenyl isomers and para-oxon are very active (5,116, 146,322,417). A similar situation exists with regard to EPN (118, 322) and a number of other thionophosphates, including methyl parathion and malathion (322).

It has been shown that nitric acid can oxidize parathion to para-oxon (178), and Cook (95,97) and Fallscheer and Cook (146) found that ultraviolet light or oxidizing agents such as *N*-bromosuccinimide or bromine water would convert parathion to para-oxon, and other insecticides containing the thiono group into much stronger inhibitors.

The metabolism of parathion has been studied by a number of workers (102,172,219,253,354) and has been reviewed by Hazleton (218). Parathion is not stored to any appreciable degree in animals and is readily metabolized to a thiophosphoric acid moiety, *p*-nitrophenol, and a conjugate of *p*-aminophenol. Tests for *p*-nitrophenol in urine have been described (288,440).

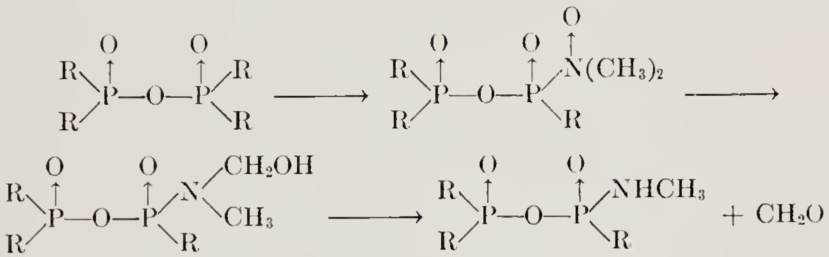
### 5. *Schradan and Dimefox*

Schradan is stable to hydrolysis in water but can be hydrolyzed by the action of alkali (221) to tetramethylphosphorodiamidic acid, $[(CH_3)_2N]_2PO_2H$, or by acid hydrolysis to dimethylamine and orthophosphoric acid. The technical product contains the three by-products shown in (IV) (215).



$R=(CH_3)_2N—$
(IV)

Schradan is metabolized in plants (77,78,80,124,201,214,221, 223,381), animals, or animal tissues (76,124,170,266), and in some insects to schradan *N*-oxide according to the scheme (V) proposed by Hartley, Heath, and their co-workers (211,212,214,223) and confirmed by Casida and co-workers (80) and O'Brien and Spencer (344).



$R = (CH_3)_2N—$
(V)

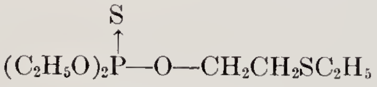
Further metabolism can also take place. In contrast to schradan, schradan *N*-oxide is a powerful cholinesterase inhibitor and is easily hydrolyzed.

The *N*-oxide can also be produced by chemical oxidation (5,74,76, 78,97,146,178,211,212,344,433). Tsuyuki *et al.* (433) have reviewed recent knowledge concerning the enzymatic and chemical oxidation products of schradan. In addition, they have shown that schradan *N*-oxide can be converted by means of alkali, acid, or heat into heptamethylpyrophosphoramide *N*-methoxide, which is also obtainable directly from schradan by peracetic acid oxidation. These products probably account for the occurrence of two metabolites in plants, as found by Hartley (212). Dimefox is also converted to a more active enzyme inhibitor by permanganate oxidation (74,76).

Ripper *et al.* (380) conducted extensive feeding experiments with schradan-treated plants and noted no toxic effects, and Martin (307) and Casida *et al.* (78) found that residues were decomposed readily so that there was a negligible hazard after about six weeks. However, Jones and Thomas (255) showed schradan to be present in honey from the nectar of plants treated four weeks previously.

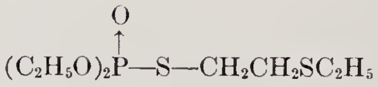
### *6. Demeton (Systox)*

Demeton is a mixture of thiono and thiol isomers (in about 2:1 ratio), the chemical, physical, and biological properties of which differ considerably (162,171,213,226,386):



$$(C_2H_5O)_2\overset{\overset{S}{\uparrow}}{P}\text{—O—}CH_2CH_2SC_2H_5$$

Thiono isomer



$$(C_2H_5O)_2\overset{\overset{O}{\uparrow}}{P}\text{—S—}CH_2CH_2SC_2H_5$$

Thiol isomer

Metcalf, March, and co-workers (163,300,301,315,324,325,326) have extensively described the behavior of these two isomers in insects, plants, and animals with the aid of radioactive compounds and paper chromatography. In the metabolism of demeton, the thiono group of the thiono isomer can be oxidized to an oxygen atom, and the thioether group in both the thiono- and thiol- forms can be oxidized to give the corresponding sulfoxides and sulfones. The metabolic products were identified by comparisons with synthetic products. The thiolphosphate sulfoxide and thiolphosphate sulfone are the important metabolites and are several times as active as the original insecticide as cholinesterase inhibitors. The toxic products are slowly hydrolyzed in the plant. Heath (220) also found several metabolites in turnips.

Dahm and Jacobson (103) found that significant amounts of residues were present in baled hay stored in a barn for one year. This is in contrast to most other workers who have found a rapid decrease to very low values in several weeks with somewhat longer persistence in waxy or oily plants (82).

### *7. TEPP (Tetraethyl Pyrophosphate)*

Tetraethyl pyrophosphate, a very toxic chemical and powerful cholinesterase inhibitor, is readily hydrolyzed by water to the innocuous diethylphosphoric acid.

### *8. Phosdrin (1-Carbomethoxy-1-propen-2-yl Dimethyl Phosphate)*

Phosdrin consists of a *cis* and a *trans* isomer in about 2:1 ratio (73). In a thorough study of Phosdrin by Casida *et al.* (79), residues were checked by bioassay, cholinesterase inhibition, radiotracer, and chromatographic methods, and possible routes of metabolism were discussed. This pesticide is somewhat volatile and is hydrolyzed to nontoxic materials. The *cis* form is 100 times more toxic than the *trans* form. After foliage treatments, there was a 90% loss of residue in two days and over 99% in four days. In general, residues are essentially dissipated within two days following insecticide application.

## III. Separation and Cleanup Procedures

After extraction of the sample to be analyzed, the pesticide usually has to be separated from the other materials in the extract. The greater the degree of separation attained without loss or undesired decomposition of the pesticide, the better will be the results of the analysis; also, a wider variety of detection techniques will then be available for the final estimation.

The *physical* methods that have been utilized for separation of the pesticide from other materials in the extract are: (a) distillation—simple, vacuum, and azeotropic, carrier or other type of codistillation; (b) chromatography—adsorption and partition; (c) partition between solvent pairs or countercurrent distribution; and (d) crystallization.

In many instances, the *interfering materials* must be *chemically* modified by a process such as oxidation, reduction, or hydrolysis (acidic, basic, or enzymatic). In some cases, cleanup is facilitated by the *chemical* changes made in the *pesticide* such as the formation of a volatile product (for example, benzene, carbon disulfide, dimethylamine, ethylene oxide, and hydrogen sulfide), or acidic or basic products. These physical and chemical processes may be used alone or in combination for the separation of the pesticide from the plant or animal extract. Only a few typical examples of isolation procedures taken from methods discussed later will be mentioned.

### A. DISTILLATION METHODS

#### *1. Steam Distillation*

In the analysis of ovex (Ovotran), Kutschinski and Luce (279) separated *p*-chlorophenol from the acidified hydrolysate and from possible interferences by means of steam distillation. Gunther and Jeppson (200), in the analysis of ovex in lemons and oranges resorted to a double steam distillation. The hydrolysate containing the sodium *p*-chlorophenate was steam-distilled to remove the interfering oils and waxes, acidified, and then the free *p*-chlorophenol was steam-distilled and determined.

### *2. Vacuum Distillation*

The analysis of schradan residues by Heath *et al.* (222) is a good example.

### *3. Carrier Distillation*

Dupée *et al.* (126) make use of carrier distillations with glycerol-ethylene glycol, or with a petroleum oil, in the analysis of dimefox residues.

## B. CHROMATOGRAPHIC METHODS

### *1. Adsorption Chromatographic Separations*

In the phenyl azide or total-chlorine procedure for aldrin, O'Donnell *et al.* (346) chromatographed a Skellysolve B extract on a mixed adsorbent. The eluant contained aldrin, and interferences were retained on the column. When the amount of extractive was too great to be directly chromatographed, a preliminary separation was made by shaking the extract with an adsorbent mixture of activated carbon, silicic acid, and Attasol. Materials high in glycerides required a preliminary saponification, which could be applied because of the alkali stability of aldrin. O'Donnell *et al.* (347) utilized similar procedures for dieldrin.

Norton and Schmalzreidt (343) found that some extracts gave interfering colors when applying the Schechter-Haller method for DDT (395). Interferences were removed by chromatography of a benzene solution of the extract on alumina. Martin and Batt (309) substituted carbon tetrachloride for benzene in order to avoid any possible color interference from the latter solvent.

Patchett (356) determined Sulphenone employing a variety of cleanup procedures. For isooctane extracts from fruit surfaces, chromatography on Magnesol was sufficient. From fruit pulp, the extract was first chromatographed on alumina and then on Magnesol;

and for extracts of animal tissues, partitioning between solvent pairs as suggested by Jones and Riddick (259) was used followed by a chromatographic separation on alumina and then on Magnesol. Dye indicators were used to indicate where fraction collection should start. The chromatographic separation on alumina served to separate the Sulphenone from interferences and the Magnesol chromatography effected complete resolution of the component sulfones present in the technical Sulphenone.

### *2. Partition Chromatographic Separations*

A chromatographic technique that has been widely used to separate fatty materials from DDT was developed by Davidow (107). In this procedure, Celite is impregnated with sulfuric acid-fuming sulfuric acid and slurried with carbon tetrachloride. When fats plus DDT in carbon tetrachloride are passed through such a column, the fats are retained on the column while the insecticide is eluted. This separation technique has been applied to other fat-soluble insecticides stable to the action of sulfuric acid, such as BHC and toxaphene.

A reversed-phase partition chromatographic procedure has been developed by Erwin *et al.* (139) to isolate pesticides from plant and animal extracts. This type of chromatography is characterized by having the immobile phase of lower polarity than the mobile phase. Chromatographic alumina coated with 15% by weight of a 2:1 petrolatum-paraffin wax mixture was found to be the best column material. The preferred eluant was aqueous acetonitrile, and it was found that all but a small fraction of interfering extractives were removed. Insecticides were divided into three groups that could be eluted separately by increasing the concentration of acetonitrile in the mobile phase successively from 40 to 60 to 75%. Insecticides successfully separated (90–100% recovery) on this type of column from spinach extracts, which are difficult to handle because of the high extractives content, included DDT, methoxychlor, lindane, chlordane, heptachlor, aldrin, toxaphene, parathion, and malathion.

### *3. Paper Chromatographic Separations*

The difficulties in the application of paper chromatography to residue analyses are, first, cleanup must be adequate in order to obtain reproducible $R_f$ values and spots that are meaningful and not too diffuse. Second, a system of solvents must be found that will give effective separations. Third, the amount of material in the desired spot on the paper, usually in the microgram or submicrogram range, must be determined quantitatively.

A number of separations of pesticides have been obtained by paper chromatography. Moynihan and O'Colla (337) separated the isomers of hexachlorocyclohexane and heptachlorocyclohexane present in technical BHC. O'Colla (345) extended this work to include chlordane, toxaphene, and DDT. Mitchell (330) and Mitchell and Patterson (331) separated many of the other chlorinated insecticides such as aldrin, dieldrin, endrin, and isodrin; Bulan and Prolan; and DDT, TDE, and methoxychlor. Gruch (190) separated DDT, parathion, and BHC, and Bennet-Clark *et al.* (33) separated 3-indoleacetic, 3-indolepropionic, and 3-indolebutyric acids and estimated the amounts by chemical and biological tests. Metcalf and March (322,323) and Gage (165) used reversed phase paper chromatography for the separation of parathion, its isomers and related compounds. March *et al.* (300) used paper chromatography for an investigation of schradan and demeton. Cook (95) separated demeton isomers, EPN, parathion, Diazinon, Chlorthion, methyl parathion, and malathion. He was also able to apply a cholinesterase inhibition test directly to developed chromatograms (96).

Considerable use of paper chromatography has been made, often in conjunction with the use of radioactive compounds in investigations of the metabolism of pesticides in plants and animals (see section on metabolism of pesticides). The metabolism of demeton and schradan, especially, has been studied in some detail (163,300,301,315,324,325, 326,377,420,433). No methods appear to have been developed employing paper chromatography for routine residue analyses.

## C. PARTITION AND COUNTERCURRENT SEPARATIONS

### 1. *Partition Separations*

Partition between two immiscible solvents is coming into wider use and is a valuable means for the concentration of pesticides from their residues and for the elimination of interferences. In general, the insecticide should have a solubility preference for one of the solvents, and extraneous or interfering materials for the other solvent. The determination of partition coefficients in one or more pairs of immiscible solvents is also used in the identification of pesticides.

This method was successfully applied by Jones and Riddick (259) to the isolation of Dilan from plant and animal tissues and dairy products employing hexane and acetonitrile. Recoveries averaged 99%. Exploratory tests with parathion, chlordane, lindane, methoxychlor, and DDT indicated that this technique could be used widely. Burchfield and Storrs (59) compared the partition of several insecticides between *n*-hexane and acetonitrile with that between *n*-hexane and *N*,*N*-dimethyl formamide. The latter combination was preferable for DDT and lindane. Claborn and Beckman (86) selected *n*-hexane and nitromethane for the separation of methoxychlor from fats. The fat preferentially dissolved in the hexane phase and the methoxychlor partitioned into the nitromethane. Kunze (278) described a cleanup procedure for the analysis of methoxychlor which combined partition of methoxychlor between hexane and acetonitrile with an alkaline saponification of fats and waxes not removed by the partition step. The cleanup was completed by chromatography to remove other interferences.

Schechter *et al.* (394) in the determination of DDT in milk and fatty materials, removed the fatty material by washing a chloroform solution of the extracted fat with sulfuric acid-fuming sulfuric acid several times. The fats dissolved in the acid phase and unchanged DDT partitioned into the chloroform layer. The chloroform phase was washed, and the solvent removed leaving a residue that could be analyzed for DDT. This method has general applicability in the cleanup of other pesticides which are not soluble in, or affected by sulfuric acid, such as toxaphene and lindane, but fails with methoxychlor and other insecticides soluble in sulfuric acid. The sulfuric acid not only directly dissolves many fats, other esters and most oxygenated compounds, but also reacts with many aromatic and unsaturated compounds, leaving only a small amount of inert material from fats and oils which probably is mainly paraffinic in nature.

### *2. Countercurrent Separations*

Countercurrent techniques have been found to be useful for the isolation of pesticides and their metabolites (see section on metabolism of pesticides). Although too involved for routine residue determinations, an application has been made in the analysis of 2,4-D (443) (see section on analysis of herbicides).

## D. CLEANUP BY CRYSTALLIZATION

In the method of Fairing and Warrington (145) for methoxychlor, the impurities crystallized from acetone to a considerable extent, leaving the methoxychlor in solution. Gunther and Blinn (198) found that DDT remained preferentially with the benzene which crystallized out when benzene extracts of avocado pulp were cooled to 0°.

## E. CLEANUP BY OXIDATION OF INTERFERENCES

Gunther *et al.* (193) determined dieldrin in oranges after oxidation of interfering materials in the extract with alkaline permanganate to readily removable alkali-soluble materials, since dieldrin is stable to this treatment. As another example of oxidation, some cleanup is attained during the nitration process in the Schechter-Haller method for DDT (395).

## F. CLEANUP BY REDUCTION OF INTERFERENCES

In the method for the determination of 2,4-dichloro-6-(*o*-chloroanilino)triazine (364), boiling with zinc is employed to reduce interfering colors.

## G. CLEANUP BY HYDROLYSIS OF INTERFERENCES

Examples of acidic or basic hydrolysis are numerous, as can be seen in methods given for many of the pesticides. Hydrolysis by lipolytic enzymes has been used to remove fat and by proteolytic enzymes to remove proteins. Clifford (90) has employed hog pancreas lipase to treat as much as 5 g. of butterfat, and Gunther and Blinn (198) have used papain to remove interfering proteinaceous material in the determination of ovex.

## H. CLEANUP BY CHEMICAL CHANGES IN THE PESTICIDE

### 1. *Formation of Volatile Products*

Carbon disulfide is formed from dithiocarbamates (88), hydrogen sulfide from Diazinon (426), ethylene oxide from Aramite (199), dimethylamine from schradan (201), and benzene from benzene hexachloride (392). These volatile products are swept from the reaction mixture and then determined.

### *2. Formation of Acidic Products*

Dichlorobenzilic acid is produced in the analysis of Chlorobenzilate (48). Ovex and Neotran can be hydrolyzed to *p*-chlorophenol, which is then determined (120,279).

### *3. Formation of Basic Products*

Many of the carbamate herbicides can be hydrolyzed to the constituent aromatic amines, which can then be determined. In the analysis of Diazinon residues, both the basic and acidic properties of the pyrimidinol moiety are employed for cleanup.

### *4. Reduction*

Reduction of a nitro group to an aromatic amine is employed in the Averell-Norris method (22) for parathion, as well as for methyl parathion and EPN.

### *5. Oxidation*

Claborn and Patterson (87) in the determination of DDT utilized oxidation of DDE to 4,4′-dichlorobenzophenone and this aided the cleanup. Blinn *et al.* (48) oxidized the dichlorobenzilic acid to 4,4′-dichlorobenzophenone in the analysis of Chlorobenzilate.

## IV. Physical Methods of Measurement

After suitable cleanup or separation procedures have been applied, the pesticide may be determined by such physical procedures as spectrophotometry in the visible, ultraviolet, or infrared regions, or radiometric determination of radioactively labeled pesticides. However, one or more chemical reactions may have to be applied before the determination by physical means.

### A. SPECTROPHOTOMETRIC METHODS

The relatively specific methods that have been developed for microgram quantities of pesticide residues depend almost entirely on spectrophotometric measurements. The infrared region of 2–15 $\mu$, the ultraviolet region of 200–400 m$\mu$, and the visible region of 400–750 m$\mu$ are usually used. All organic molecules show absorption in the infrared. Since the infrared absorption spectrum for a molecule is often unique, it frequently affords a good means of identification when the spectrum of the known material is available for comparison. The visible and ultraviolet regions are of greater interest for the analysis of pesticide residues since the molar absorptivities are usually of a much higher order of magnitude than those in the infrared; thus methods of greater sensitivity may be obtained. If the compound has an absorption band of high enough intensity in the ultraviolet region, or if one can be generated, this may be made the basis of an analytical procedure. Cleanup procedures must be used and interfering impurities in the solvents must be removed. In the visible region, since most pesticides are not colored, a chromophoric structure must be created or introduced into the pesticide molecule before it can be determined spectrophotometrically. For highest accuracy and precision, spectrophotometers should be used; however, filter photometers and colorimeters are usually less expensive and simpler to operate. Nephelometric and fluorimetric methods can be employed but these have not been widely applied for residue analyses. To help correct for background interferences, mathematical corrections of the Morton and Stubbs type (336,352) and graphical baseline techniques (173,224,458) are useful.

### B. ELECTROMETRIC METHODS

A number of electrometric techniques can be utilized for the final determination of an ionic product such as chloride ion, derived from the pesticide. These include direct potentiometric measurements, potentiometric titrations, amperometric titrations, and coulometric measurements.

Polarographic methods are usually subject to interferences from plant extracts, and many of them lack the sensitivity needed for residue analysis. However, there seems to be no reason why, with adequate cleanup procedures and the use of small-scale polarographic cells, more use could not be made of this type of analysis for residues

### C. RADIOACTIVE METHODS

Radiometric and radioautographic techniques can be employed with radioactively labeled compounds to study the composition, isomerism, and chemical reactions of pesticides, and their absorption, translocation, breakdown, and metabolism in plants, insects, and other animals. Furthermore, these radioactive compounds can be used in research on residues for following preanalysis factors, cleanup methods, recoveries, and losses at each stage of an analytical procedure.

Demeton residues have been investigated in a thorough manner by means of radioactive tracers by Metcalf, March, and co-workers (163,300,301,315,324,325). Demeton residues as low as 0.01 p.p.m. could be detected. These amounts could not have been determined precisely by other than tracer techniques.

Metcalf (315) outlined procedures used in studies with radioactive schradan and demeton in which 1 $\gamma$ or less of tracer could be readily and reliably detected; with a kilogram sample, a sensitivity of 0.001 p.p.m. is obtainable whereas most colorimetric methods are rarely reliable to 0.1 p.p.m.

Dahm (101) made a thorough survey of the preparation and application of radioactive tracers to insecticide research and Metcalf (315) illustrated the use of radiotracers in the study of systemic insecticides.

## V. Nonspecific Analytical Methods

When a pesticide contains an element such as mercury, lead, arsenic, copper, phosphorus, sulfur, or the halogens, these may be determined by a suitable procedure after combustion or wet or dry oxidation. For halogenated compounds, reduction with sodium or zinc, or reaction with bases, is often used. Where the pesticide inhibits an enzyme system, use may be made of this property such as in the cholinesterase inhibition methods used for many of the organophosphorus compounds.

### A. ORGANIC HALOGEN METHODS

#### *1. The Decomposition of Organic Halogen Compounds by the Sodium-Alcohol Method*

This method was first described by Stepanow (418). The organic halide was dehalogenated by refluxing with sodium in ethanol. Tightly bound halogens, as in vinyl-type and aromatic linkages, may not be completely reduced because of the low reflux temperature and the rapidity of reaction between sodium and ethanol. The modifications that have been introduced usually substitute a less reactive alcohol for ethanol. Umhoefer (434) found that secondary alcohols, such as isopropyl and isobutyl, were particularly effective for the decomposition of organic halogen compounds with sodium. Carter and co-workers (68,69,70) applied this method with sodium in isopropanol to chlorine-containing organic insecticides. The insecticide was extracted from the sample with a low boiling solvent, the solvent was evaporated, the residue reflux with isopropanol and metallic sodium, and the chloride ion determined by a Volhard titration, or preferably potentiometrically with standard silver nitrate solution. The sensitivity of chloride determination with these titration techniques is about 75–100γ. In general, reagent blank values are appreciable, and if more sensitive chloride methods are tried, the large quantities of salts formed by neutralization of the alkaline solution after the reduction may interfere. Under favorable circumstances, results reliable to 1.0 p.p.m. may be obtained. Ely *et al.* (135) considered 1.0 p.p.m. of aldrin in milk as the lower limit of validity. The use of this method for residues of DDT is described in the Methods of Analysis of the Association of Official Agricultural Chemists (17, pp. 406–415).

Phillips and DeBenedictis (363) have modified the sodium reduction method so that more sensitive methods for determining chloride ion may be utilized. A preliminary cleanup of the strip solution is used to reduce the amount of extractives prior to sodium reduction to no more than 100 mg. The reduction itself is carried out for 1 hour with 1 g. of sodium in 10 ml. of isobutanol. With these smaller quantities of reagents, blanks are reduced considerably. The reflux temperature of isobutanol is higher than the reflux temperature of isopropanol and more complete dechlorination is obtained with materials difficult to reduce completely such as dieldrin. Chloride ion is determined by amperometric titration. The sensitivity is about 1 γ of $Cl^-$ in 10 ml. of solution and reagent blanks run about 15–20 γ of chloride.

Liggett (289) described the use of sodium biphenyl in a mixed solvent. This may prove to be useful but it does not seem to have been applied to residue analysis.

### *2. Combustion Methods*

Combustion techniques have been described that avoid interference due to the use of sodium and the formation of salts by neutralization in the sodium-reduction method. Peters *et al.* (358) described a detailed combustion procedure for both sulfur and chlorine, and

Agazzi *et al.* (1) adapted the method to highly chlorinated pesticides. They described three procedures: (a) *Macro procedure.* Residues are burned in an air stream, the air being first purified by passage through a quartz tube at 700°–800° in order to convert any organic halides which may be present to halogen acids which are absorbed in dilute alkali. This purified air is then used to burn the sample in a second combustion furnace and the hydrochloric acid formed is absorbed in 0.2% sodium carbonate solution. The chloride is measured amperometrically. (b) *Semimicro method.* In preliminary cleanup stages the extractives may be reduced to 50 mg. or less; in such cases the samples may be burned in a simplified apparatus. (c) *Lamp method.* Animal fat was found to be essentially free from organic chlorine but contained sufficient inorganic chloride to preclude application of the quartz tube combustion method. To avoid the use of cleanup methods to remove inorganic chlorides a lamp combustion method was developed which allows the selective determination of organic chlorine in the presence of inorganic chloride. Blanks using 1 g. of fat were approximately 22 p.p.m. of chloride. The lamp method is an adaptation of the A. S. T. M. method for the determination of sulfur in petroleum products (12). In all combustion procedures, any small amounts of free halogen which may be formed can be reduced to the halide by the addition of a small amount of hydrogen peroxide to the alkaline absorbing liquid. The macro quartz tube method has the greatest general utility since the sample size may range from several milligrams to a few grams. In this procedure (1), aldrin and dieldrin blanks equivalent to about 5 $\gamma$ of aldrin or dieldrin were found. Ewart *et al.* (141) have applied this combustion method to dieldrin in citrus fruits. Ely *et al.* (134) have analyzed dieldrin in milk and O'Donnell *et al.* have applied this method to both aldrin and dieldrin in a wide variety of crops (346, 347). Gunther and Blinn (198) report the use of this method for Aramite, DDT, ovex, and *p*-chlorophenyl phenyl sulfone.

A vertical, quartz packed furnace has been described by Hudy and Dunn (243). The air for combustion is drawn through the system by an aspirator and is purified by preburning in a separate furnace. There is no strict limit to sample size since the sample as such, or dissolved in a halogen-free solvent, is introduced dropwise by means of a dropping funnel into the combustion area. The unit is particularly useful for halogen-containing pesticide residues which cannot be separated readily from extracts. The method has been applied successfully to vegetable oils, animal fat, and butter. Carter and Hubanks (67) have determined aldrin, dieldrin, toxaphene, and BHC in this manner. A combustion method for lindane in flour has been described by Krauze *et al.* (276). White *et al.* (447) described an automatic combustion apparatus for sulfur and halogen which is an elaboration of the apparatus described by Peters (358) and Agazzi (1).

### *3. Dehydrohalogenation and Dehalogenation Methods*

In addition to methods for the determination of total chlorine, procedures have been developed for removing part of the chlorine present in a molecule by treatment with alkali (although in some instances all of the chlorine is removed under these conditions). This is usually accomplished by refluxing with aqueous or alcoholic alkali, or alkoxides in an alcohol. Dehydrochlorination or substitution may take place depending on the nature of the molecule and the experimental conditions but some rough generalizations may be made. Reaction occurs readily if *trans* elimination of hydrogen chloride is possible but may be slight or nil in certain ring structures where dehydrohalogenation could lead to the formation of highly strained systems. In substitution reactions, vinyl, bridgehead, and aromatic halogen atoms are particularly inert.

These methods, when applicable, have the advantage of extreme simplicity. In addition, the difference in reactivity of halogen atoms under different reaction conditions may yield information unobtainable by total chloride methods. LaClair (283) described a method for the determination of the gamma isomer in the technical product based on differences in dehydrochlorination rates of the isomers of BHC to trichlorobenzene. Klein (270) developed a method for the beta isomer of BHC based on the fact that it is the most resistant to alkaline dehydrochlorination. Barlow (28) determined BHC in the blood of cattle by the dehydrohalogenation of BHC to trichlorobenzene with ethanolic potassium hydroxide and determination of the chloride ion formed. Neal (340) and Gunther (192) proposed a method for DDT based on dehydrochlorination with alcoholic potassium hydroxide to liberate one atom of chlorine per molecule of DDT.

Howard (242) developed a method for BHC in foodstuffs based on dehydrohalogenation to isomeric trichlorobenzenes with ethanolamine.

Chloride equivalent to 200 γ of BHC was determined by Volhard titration and chloride ion equivalent to as little as 20 γ of BHC by a turbidimetric method. Hornstein (236) has suggested the use of zinc reduction, by percolation of an acidified solution containing the chlorinated pesticide through a granulated zinc column which removes part or all of the chlorine as chloride ion.

### *4. Methods for Determination of Halide*

As methods for converting the chlorine in organic pesticides to chloride ion have become refined, the sensitivity of the methods for determining chloride ion has kept pace. The Volhard titration method has been replaced to a large extent by potentiometric, amperometric, and coulometric methods.

The sensitivity of a potentiometric titration for chloride ion is about 50 γ and this method is widely used for amounts of chloride ion above this value. The titrations may be made with automatic titration equipment. Direct potentiometric methods have also been described in which the potential of a silver-silver halide electrode in the solution to be analyzed is measured against a standard electrode and this value is related to chloride ion concentration. Blaedel *et al.* (42) developed a direct potentiometric method which was sensitive to about 0.7 γ of $Cl^-$ in 10 ml. of solution, using a silver-silver chloride electrode in a half-cell containing the unknown solution and another such electrode in a half-cell of known chloride concentration. The theory of the method is discussed by Furman and Low (164). Chanin (81) used a mercury-mercurous sulfate reference electrode and a silver-silver chloride electrode immersed directly into the unknown. A chloride concentration of as low as 3.5 γ per ml. could be determined in this manner. Helmkamp *et al.* (225) used this same electrode system under more carefully controlled conditions. A concentration range of about 0.02 to 10,000 p.p.m. was obtainable. This method was combined with the combustion technique of Agazzi *et al.* (1) to determine aldrin, Aramite, BHC, chlordane, chlorobenzilate, *p*-chlorophenyl *p*-chlorobenzene sulfonate, DDT, dieldrin, endrin, heptachlor, isodrin, lindane, methoxychlor, Sulphenone, and toxaphene. Each of these insecticides was statistically determinable at the 0.1 p.p.m. level.

For chloride methods sensitive below the 50 γ level, amperometric methods are most frequently utilized. In amperometric titrations, the current at a constant applied voltage is observed while known quantities of a titrating agent are added. The indicator electrode commonly used for this system is a rotating platinum electrode and the reference electrode may be a saturated calomel electrode. Laitinen *et al.* (281) have described an amperometric titration of chloride, bromide, and iodide. Agazzi (1) adapted this method to chloride ion resulting from the combustion of chlorinated insecticides.

Chloride determinations may be made coulometrically with electrolytically generated silver ion. Methods for chloride, bromide, and iodide described by Lingane (291) and for iodide by Kowalkowski *et al.* (275), should prove useful. Coulometric titration techniques can be made automatic for micro amounts of halide. An additional advantage is the absence of standardized titrants.

Colorimetric and nephelometric methods for halide have been suggested but are not widely used. However, Gordon (184) has proposed a colorimetric method for chloride ion liberated from chlorinated organic insecticides based on the Conway (92) technique in which chloride ion is oxidized to chlorine by permanganate in the outer chamber of a Conway microdiffusion cell. The chlorine then diffuses and decolorizes a dye solution in the inner cell chamber. The decrease in absorption of the dye measured photometrically is proportional to the chloride.

### B. ENZYMATIC METHODS OF ANALYSIS

The enzymatic methods of analysis for pesticides have been limited almost entirely to organophosphorus and carbamate insecticides. The inhibition of the cholinesterase (ChE) enzyme system of animals is one of the outstanding characteristics of these compounds and probably accounts for their high mammalian toxicity, although some doubt has been shed on the hypothesis that this is the basic reason for toxicity to insects (234,294,295). Many of these compounds and certain of their metabolites and reaction products are such powerful ChE inhibitors that methods of analysis have been based on this property. While the natural substrate of "true" or "specific" ChE is acetylcholine (ACh), neither the natural substrate, function or importance of pseudocholinesterase (nonspecific cholinesterase) is known (20,29). Furthermore, the ChE systems of different species (20,32,71,319,320) and even from different tissues (20,71,72) differ. Blood yields ChE preparations from serum and from erythrocytes which are quite different (20). ChE preparations may be mixtures of true and nonspecific cholinesterases together with other esterases and may vary considerably in their effects on different substrates, with different inhibitors, and even with the concentration of the substrates (4,20,71).

A number of enzymes with esterase action, such as chymotrypsin, trypsin, certain insect esterases, and other enzymes can also be inactivated by organophosphates (4,317). In the case of diisopropyl fluorophosphate (DFP) and some other organophosphates, Jansen and co-workers (247–250) have shown that a 1:1 stoichiometric reaction takes place between $\alpha$, $\beta$, or $\gamma$ chymotrypsin (chymotrypsin is both a proteinase and an esterase) and the inhibitor resulting in a crystallizable inactivated enzyme containing the diisopropyl phosphate radical from DFP. A similar reaction with chymotrypsin occurs with TEPP (153) and with para-oxon (216).

ChE hydrolyzes its normal substrate, acetylcholine (ACh), into acetic acid and choline (other substrates may be used also):

$$CH_3COOCH_2CH_2\overset{+}{N}(CH_3)_3 + H_2O \xrightarrow{\text{ChE}} CH_3COOH + HOCH_2CH_2\overset{+}{N}(CH_3)_3$$

In analytical methods, an excess of ChE is first allowed to react with the inhibitor. After the incubation period, the uninhibited ChE left hydrolyzes the ACh:

$$(1)\quad \text{Excess ChE} + \text{inhibitor} \longrightarrow \text{inhibited ChE} + \text{uninhibited ChE}$$

$$(2)\quad \text{Excess ACh} + \text{uninhibited ChE} \longrightarrow [\text{ACh}\cdot\text{ChE}] \longrightarrow$$
$$CH_3COOH + \text{Choline} + \text{unhydrolyzed ACh} + \text{ChE}$$

The greater the amount of uninhibited ChE left after reaction with the inhibitor, the greater will be the amount of acetic acid and choline produced, and the less will be the amount of ACh unhydrolyzed after the specified time of reaction has elapsed. Since enzyme preparations vary, the source and method of preparation of the enzyme must be carefully specified in analytical procedures.

Either the amount of acetic acid or choline produced or the amount of ACh left after hydrolysis can be determined. The acetic acid may be determined by measuring the change in pH of the solution, by microtitration, or by a Warburg manometric measurement of the carbon dioxide liberated from a bicarbonate solution. Some methods depend on the colorimetric determination of the ACh remaining after hydrolysis.

In many cases, the organophosphorus compounds, carbamates, or other enzyme inhibitors can be changed *in vivo* or *in vitro* to products some of which are considerably less active and some considerably more active than the original insecticide. Thus, methods based on ChE inhibition are nonspecific. However, there are differences in the effects of some insecticides on different enzyme systems and it might be possible by selection of the proper enzyme systems and substrates to increase the specificity of enzymatic methods. Additional specificity can be obtained by the use of paper or column chromatographic techniques, partition between solvents, and countercurrent methods. The determination of hydrolysis rate constants, partition ratios, and Rf values are of considerable use for identification purposes. Even though they have serious limitations, ChE inhibition methods are of great value and some idea of the possible hazard from residues may be obtained with them. Indeed, some Food and Drug Administration tolerances under the Miller law specify that a ChE inhibition method be employed for residue determinations.

Metcalf (317) and Fallscheer and Cook (146) give inhibition values for inhibitors which are of general interest. Fleisher *et al.* (155) have suggested that stable, enzymatic standards of bovine red cell ChE on filter paper disks be used as standards for ChE activity determinations, for comparison of different methods, or as controls in studying the reliability of methods. A fairly pure cholinesterase product is available commercially (284).

### *1. Electrometric ChE Methods*

Giang and Hall (178) have adapted Michel's electrometric method (327) to the enzymatic determination of organophosphorus insecticides. They extract the plant material with ether, wash with 10% sodium bicarbonate solution and saturated sodium chloride solution, make the ether solution to volume while cold, evaporate an aliquot to dryness, and incubate the aliquot with a standard ChE solution (purified bovine- plus human serum-ChE) and a standard buffer at 25° for 30 minutes. A standard acetylcholine chloride solution is added and after a fixed time interval the pH change is measured. The per cent inhibition is calculated from the pH change and is related by a standard curve to micrograms of the inhibitor. Their method was developed mainly for tetraethyl dithiopyrophosphate residues although tetraethylpyrophosphate, EPN, and demeton could also be determined. Methyl parathion and schradan as such were too weakly inhibitive to be determined (although they no doubt could be if oxidized). Although parathion is a comparatively weak inhibitor of ChE, these authors have shown that it may be oxidized by strong nitric acid to para-oxon, a powerful inhibitor which can be measured in hundredths of a microgram. The chlorinated hydrocarbon insecticides, rotenone, pyrethrum, and a number of plant extracts did not interfere. Hensel *et al.* (227) have utilized a similar procedure for demeton residues and Frawley *et al.* (156) for a number of organophosphorus compounds in pharmacological studies.

Hamblin and Marchand (204) and Hamblin and Golz (203) have described a microelectrometric modification of Michel's method.

### 2. Titrimetric ChE Methods

Brown and Bush (55) used a titrimetric ChE method for parathion, and a microtitrimetric method based on the work of Glick (182) has been employed by Metcalf and March (319) for parathion and its derivatives. Other modifications of Glick's procedure have also been employed (8,23).

### 3. Manometric ChE Methods

A number of workers have employed Warburg manometric ChE methods based on the amount of carbon dioxide liberated from sodium bicarbonate added to the reaction medium. As examples, Metcalf and March (319) have studied parathion and its derivatives and also schradan and its metabolites (321). DuBois and Cotter (123) have used the manometric procedure of DuBois and Mangun (125) to determine Dipterex in milk and Casida *et al.* (77,78) and DuBois *et al.* (124) employed a manometric method for a study of the absorption, metabolism, and pharmacology of schradan. Brauer (53) used Ammon's manometric method (13) for a variety of organophosphorus compounds and Lord (292) investigated residues of demeton, demeton-S, parathion, and dimefox employing a manometric technique with rabbit serum ChE and roach ChE. Casida *et al.* (75,78) have also utilized the enzyme chymotrypsin for manometric enzyme inhibition studies of schradan and its metabolites.

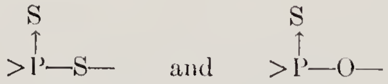
### *4. Colorimetric ChE Methods*

Hestrin (229) described the use of hydroxylamine in the determination of ACh and other carboxylic acid esters. The reaction for ACh (the choline ester of acetic acid) is:

$$CH_3COOCH_2CH_2\overset{+}{N}(CH_3)_3 + H_2NOH \longrightarrow CH_3CONHOH + HOCH_2CH_2\overset{+}{N}(CH_3)_3$$

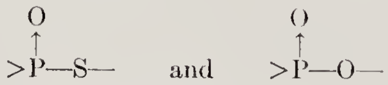
The acethydroxamic acid forms a colored complex with $Fe^{+++}$ which can be measured at 520–540 mμ. Metcalf (316) utilized Hestrin's reaction for ACh to develop a colorimetric method for the microestimation of human plasma- and red cell-ChE particularly useful for the detection of poisoning by organophosphorus insecticides. This procedure has also been adapted to whole blood (154). Cook (94) made Metcalf's technique the basis of his procedure for residues of ChE-inhibiting pesticides, employing demeton (Systox) as a standard and measuring the absorbance of the colored ferric acethydroxamic acid complex at 540 mμ.

Fallscheer and Cook (146), employing bromine water as an oxidant together with a modification of the ferric acethydroxamate method (94), showed that some insecticides containing the thiono group—parathion, Diazinon, malathion, methyl parathion, Chlorthion, and EPN—are converted by oxidation into much more potent *in vitro* inhibitors. In these cases, the



$$>\overset{\overset{S}{\uparrow}}{P}—S— \quad \text{and} \quad >\overset{\overset{S}{\uparrow}}{P}—O—$$

structures are probably oxidized to



$$>\overset{\overset{O}{\uparrow}}{P}—S— \quad \text{and} \quad >\overset{\overset{O}{\uparrow}}{P}—O—$$

Pure parathion is converted to para-oxon with a resultant increase in inhibitory action of 9000 times. Parathion, para-oxon, and Diazinon may be detected in several thousandths of a microgram amounts, the others in tenths of a microgram amounts. The procedure was suggested for use in the analysis of residues.

A number of other colorimetric procedures have been utilized such as that of Rider *et al.* (378) which depends on the hydrolysis of the substrate phenyl benzoate to give phenol which is determined colorimetrically after coupling with Du Pont Naphthanil Diazo Red B, and the method of Jansen *et al.* (249) which depends on estimation of the *o*-nitrophenol liberated from *o*-nitrophenylacetate by the action of citrus acetylesterase. Field tests for ChE in blood have also been developed (112,290).

### 5. *Other Enzymatic Methods*

Torda and Wolff (430) have shown that DDT inhibits carbonic anhydrase. Keller (261) made this the basis of a quantitative method in which as little as 0.2 microgram of DDT could be determined but this procedure was not applied to any residues. Cook (93) indicated that enzymatic methods might possibly be developed for benzene hexachloride, sodium fluoroacetate, and other fluorine-containing compounds.

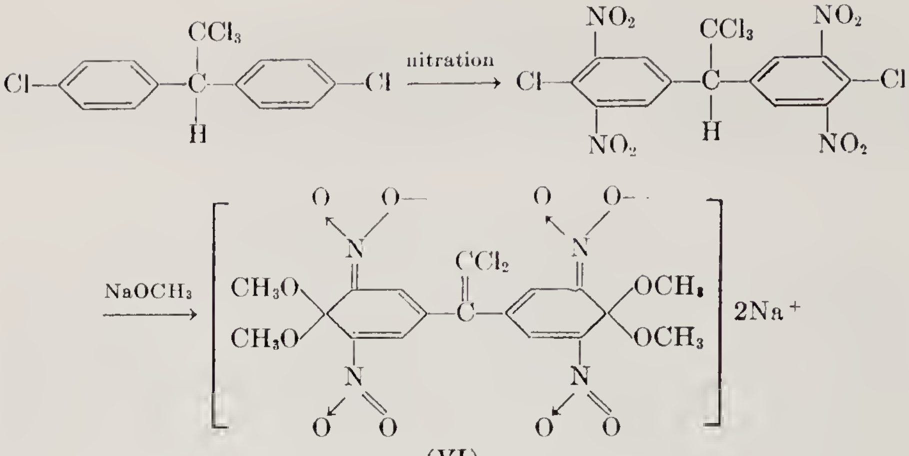
## VI. Chlorine-Containing Insecticides and Acaricides

### A. DDT

DDT is the technical product that contains 75–80% of *p,p'*-DDT and 20–25% of *o, p'*-DDT with small amounts of other products. The symbols and chemical names for a number of related compounds are *p,p'*-DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane); *o,p'*-DDT(1,1,1-trichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl)-ethane); *p,p'*-TDE(DDD)(1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane); *p,p'*-DDE(1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene); *p,p'*-DDA(bis-(*p*-chlorophenyl)acetic acid); and DBP, 4,4'-dichlorobenzophenone.

Schechter *et al.* (395) have described a method for DDT that depends on its intensive nitration to polynitro derivatives and the production of intense colors upon the addition of methanolic sodium methylate to a benzene solution of the nitration products. *p,p'*-DDT and *p,p'*-TDE give blue colors with maximum absorption at 598 mμ. *o,p'*-DDT gives a violet-red color with absorption peaks at 590 mμ and 511 mμ. Degradation products of DDT, such as DDE and DDA, yield red colors with maxima at about 420 mμ and 540 mμ.

Schechter *et al.* (391,395) found that the major nitration product formed from *p,p'*-DDT was 2,2-bis(4-chloro-3,5-dinitrophenyl)-1,1,1-trichloroethane. The reaction of the nitrated *p,p'*-DDT with sodium methylate dehydrochlorinates the compound, replaces the aromatic chlorine atoms by methoxy groups, and finally forms a colored complex with sodium methylate. The overall course of the reaction may be represented by equation (VI).



(VI)

This final product shown is actually just one contributing form to the many that can be written for the resonance hybrid. Similar reactions can be written for the *o,p′*-isomer and for TDE.

The degradation products of *p,p′*-DDT, such as *p,p′*-DDE, *p,p′*-DDA, and 4,4′-dichlorobenzophenone, are readily nitrated to 3,3′,5,5′-tetranitro-4,4′-dichlorobenzophenone in the nitration step. With sodium methylate this compound gives a red color with absorption maxima at about 420 mμ and 540 mμ.

The method can determine as little as 10 γ of DDT, or even less if smaller volumes of the final solution are used.

Technical DDT contains 75–80% of *p,p′*-DDT and 20–25% of *o,p′*-DDT. Both isomers when carried through the analytical procedure have absorption peaks around 590–600 mμ but the molar absorptivities are different. To resolve the system, use is also made of a second peak for the *o,p′*-isomer at about 510 mμ. Two standard curves are prepared, one for each isomer, and the amounts of the two isomers may be calculated by the procedure outlined by Knudsen *et al.* (271). Clifford (89) described this procedure in detail for DDT and it is a part of the procedure for DDT in the Official Methods of Analysis of the Association of Official Agricultural Chemists (17, pp. 406–415). In routine work resolution of the mixture is not necessary. A single standard curve may be prepared using a sample of technical DDT or a mixture of 3 parts of *p,p′*- and 1 part of *o,p′*-DDT, and spectrophotometric readings may be made at about 595–600 mμ. Although one can determine *o,p′*-DDT and *p,p′*-DDT by treating them as a two component system, resolution of the nitrated products from mixtures of *o,p*′-DDT, *o,p*′-DDE, *p,p*′-DDT, and *p,p*′-DDE probably is not feasible.

Modifications of the method have been made to speed up the procedure for routine application to large numbers of samples. Downing and Norton (122) substituted benzene for ether in the extraction of the nitrated product, thus avoiding an evaporation step, since the color may be developed directly in benzene. Iling and Stephenson (244) and Allessandrini (6) substituted alcoholic potassium hydroxide for sodium methylate. Iling and Stephenson (244) also used ammoniacal hydroxylamine as the base. Amsden and Walbridge (14) obtained a yellow color by the substitution of isopropylamine for sodium methoxide and Berck (34) increased the sensitivity of the method by the use of microcells having a capacity of less than 1 ml. Pearce *et al.* (357) determined DDT and related substances (particularly DDE) in human fat. A sulfuric acid-Celite column was used to isolate DDT and related products from carbon tetrachloride extracts of fats. DDA and DBP (4,4′-dichlorobenzophenone) were retained on the column, whereas DDE and DDT appeared in the eluate. After color development the spectrophotometric resolution of such a system was simplified by assuming the eluant to contain a mixture of *p,p*′-DDT and *p,p*′-DDE. Details of the procedure are given in a Public Health Service Bulletin (436). Walker *et al.* (441) determined the DDT and DDE content of prepared meals using essentially the same procedure (436). Mattson *et al.* (311) separated the DDE from DDT by chromatography of the nitration products on alumina using acetone as the developing solvent. The tetranitrodichlorobenzophenone (from DDE) was retained quantitatively on the column while the nitrated DDT isomers were quantitatively eluted. The tetranitrodichlorobenzophenone could not be removed completely from the column regardless of elution procedure, and only 65% recovery could be obtained.

Stiff and Castillo (419,420) have described an empirical method for the determination of DDT. They found that when DDT is heated in a pyridine solution containing xanthydrol (9-xanthenol) and solid potassium hydroxide, a red color develops which is proportional to the amount of DDT present. The red color can be measured at 510 m$\mu$, at which wavelength both the *o,p*′- and *p,p*′-isomers of DDT give the same amount of color. The reaction will detect as little as 10 $\gamma$ of DDT. The chemistry of the reaction has not been studied. In using this test, water and alcohol must be absent since they inhibit color formation. In addition chloroform and certain other halogenated organic compounds must also be absent since they too may give a color with pyridine and alkali. Claborn (85) found that the method was specific for DDT in the presence of TDE and could be used to distinguish between the two. DDE reacted in the same manner as DDT and gave the equivalent amount of color. Claborn also showed that at least 0.2% water in the pyridine was necessary for color development while more than 0.5% was detrimental.

Fahey and Rusk (144), in a comparative study of the determination of DDT on corn, found the Stiff-Castillo procedure more rapid and simple and nearly equal in precision and accuracy to the Schechter-Haller method. Claborn and Patterson (87) described a dinitrophenylhydrazine method for DDT. DDT is converted by dehydrohalogenation with alkali to DDE which is oxidized with chromic acid in glacial acetic acid to dichlorobenzophenone. The dichlorobenzophenone is converted to the 2,4-dinitrophenylhydrazone, which yields a color with alkali (maximum at about 530 m$\mu$). The absorption peaks and absorptivities for the *p,p′*- and the *o,p′*-isomers carried through these reactions are almost identical.

Bailes and Payne (25) described a Friedel-Crafts type reaction for DDT. Both *p,p′*- and *o,p′*-DDT when dissolved in benzene and heated with anhydrous aluminum chloride gave an orange color. This method is subject to many interferences. Bradbury *et al.* (51) developed a colorimetric method for the *p,p′*-isomer of DDT based on heating this isomer with hydroquinone in the presence of concentrated sulfuric acid and diethyl sulfate at 100°.

Herriot (228) described a spectrophotometric method for DDT based on dehydrochlorination to DDE and measurement of the absorbance at 250 m$\mu$; DDT absorbs only slightly at this wavelength.

### B. METHOXYCHLOR

In the absence of DDT and related compounds, the Stiff-Castillo method for DDT may be used to determine methoxychlor (132). Fairing and Warrington (145) have, however, described a specific method for methoxychlor that is more sensitive. Alcoholic potassium hydroxide is used to dehydrohalogenate methoxychlor to 1,1-dichloro-2,2-bis(*p*-methoxyphenyl)ethylene. The ethylenic compound on treatment with 85% sulfuric acid produces a red-colored complex with an absorption maximum at 555 m$\mu$. No other insecticide appears to interfere and as little as 1$\gamma$ of methoxychlor may be determined by this method. The intensity of color produced is dependent upon the sulfuric acid concentration so that a standard of known methoxychlor content should be run with each set of samples. The nature of the color complex is unknown; however it is likely that the ethylenic bond in conjugation with the methoxyphenyl group promotes the formation of a highly colored carbonium ion in the presence of concentrated sulfuric acid.

Claborn and Beckman (86) found that methoxychlor could be nitrated to the tetranitro derivative when fuming nitric acid alone was used instead of a nitrating mixture containing concentrated sulfuric acid. The nitrated product gave a characteristic blue color that could be measured at 590 m$\mu$ after treatment with sodium methylate. Although the blue color is not distinguishable from that produced by the tetranitro derivative of *p,p'*-DDT (because the same product is formed—see under Schechter-Haller method for DDT), the nitration procedure makes the method specific for methoxychlor since fuming nitric acid alone produces only the dinitro derivative of DDT (202). Methoxychlor is nitrated more easily than DDT because of the presence of nucleophilic methoxy groups.

Jennings and Edwards (252) utilized the ultraviolet absorption maximum at 230 m$\mu$ for concentrations of 0.04 to 0.14 g. of methoxychlor per liter in cyclohexane extracts of treated paperboard.

## C. DIPHENYLMETHANE-TYPE COMPOUNDS RELATED TO DDT

### *1. TDE (DDD, Rhothane, 1,1-Dichloro-2,2-bis(p-chlorophenyl)ethane)*

The Schechter-Haller method for DDT (395) can also be applied to TDE.

### *2. DFDT (1,1,1-Trichloro-2,2-bis(p-fluorophenyl)ethane)*

The colorimetric methods for DDT are applicable.

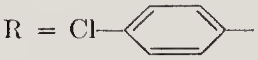
### *3. DMC (Dimite, 4,4'-Dichloro-alpha-methylbenzhydrol)*

Grummitt *et al.* (191) described macro and micro methods for this material. The micro method for residues is similar to the Schechter-Haller method for DDT and is satisfactory in the range 100 $\gamma$ to 3.0 mg. It is likely that during the extraction step, DMC is dehydrated to DDE. Absorption measurements are made at 530 mμ. The absorption curves of the final colors produced by the ethylene derivative (which is DDE) and DMC are the same since tetranitrodichlorobenzophenone is produced from each.

### *4. Chlorobenzilate (Ethyl 4,4′-dichlorobenzilate)*

Blinn *et al.* (48) described a method in which this compound is hydrolyzed to *p,p′*-dichlorobenzilic acid, then oxidized to *p,p′*-dichlorobenzophenone by chromic acid in acetic acid. The ultraviolet absorption of the ketone may be measured at 264 mμ or for greater specificity the ketone may be converted to the 2,4-dinitrophenylhydrazone and measured at 510 mμ [this is similar to the method of Claborn and Patterson for DDT (87)]. As little as 15 γ of Chlorobenzilate in 3 g. of citrus extractives can be determined in this manner. The reaction to yield the benzophenone can be written as:

$$R_2C(OH)COOC_2H_5 \xrightarrow{\text{alkali}} R_2C(OH)COONa \xrightarrow[\text{HAc}]{CrO_3} R_2CO$$

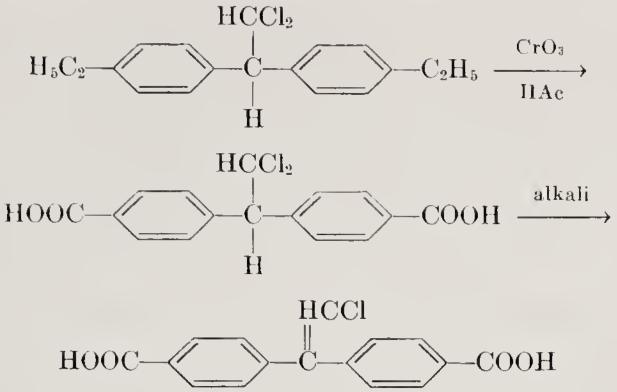


A minimum of cleanup is necessary in this method because of the nature of the steps involved. Recoveries of Chlorobenzilate ranged from 80 to 103%.

Harris (207) reported that the Schechter-Haller method could be used for Chlorobenzilate. The nitrated product upon treatment with sodium methylate yields a red product (538 mμ maximum). The method has been developed further so that this compound can be determined in the presence of DDT. The Chlorobenzilate is saponified to *p,p′*-dichlorobenzilic acid followed by extraction to remove nonacidic DDT-degradation products. Acidification liberates the *p,p′*-dichlorobenzilic acid, which is then carried through the Schechter-Haller procedure. The acid gives the same red complex as the ester. The method has been applied to several fruit crops.

### *5. Perthane (1,1-Dichloro-2,2-bis(p-ethylphenyl)ethane)*

Stanley and Jackson (415) have described a procedure involving oxidation by chromic acid in glacial acetic acid to a dicarboxylic acid followed by dehydrohalogenation hydrolysis of the acid to the ethylenic derivative, the absorbance of which is measured in the ultraviolet at 264 mμ. The reactions are as follows:

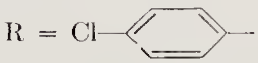


Stanley *et al.* (414) used a modification of the Fairing and Warrington method for methoxychlor (145) to determine Perthane residues. The method is based on the dehydrohalogenation with alcoholic potassium hydroxide of Perthane to the ethylenic derivative followed by reaction with strong sulfuric acid to give a color with an absorption maximum at 495 mμ. The optimum sulfuric acid concentration is 96%. Methoxychlor does not interfere materially, since its maximum absorption is at 550 mμ.

### 6. *Kelthane (1,1-bis(p-Chlorophenyl)-2,2,2-trichloroethanol)*

A method for residues of this compound has been described (383). Treatment with 50% sodium hydroxide solution results in the formation of chloroform, which is determined by the Fujiwara reaction (161). The characteristic red color is measured at 535 mμ. Materials that give a positive Fujiwara test will interfere. The reaction may be written as follows:

$$R_2C(OH)CCl_3 + NaOH \longrightarrow R_2CO + CHCl_3$$



### 7. *Dilan (Bulan plus Prolan)*

Dilan consists of 2 parts of technical Bulan (2-nitro-1,1-bis(*p*-chlorophenyl)butane) and 1 part of technical Dilan (2-nitro-1,1-bis-(*p*-chlorophenyl)propane). Jones and Riddick (258) developed a method for the analysis of these materials based on the fact that a primary or secondary nitro group may exist in equilibrium with its tautomeric aci-nitro form. The aci-nitro form produced by treatment with alkali reacts with ferric chloride to give a red complex (470 mμ maximum). Scott and Treon (398), who originally studied this reaction, reported that the colors obtained from secondary nitroparaffins fade rapidly in aqueous solution. The substitution of alcohol for water by Jones and Riddick apparently stabilizes the complex. Fifty micrograms can be determined with an error of not more than ±10 $\gamma$, and as little as 10 $\gamma$ can be detected. The method is a general one for nitroalkanes and other materials of this type may interfere.

### 8. *Chlorbenside* (*p-Chlorobenzyl p-Chlorophenyl Sulfide*)

The analytical method developed for this material by Higgons and Kilbey (230,231) is similar to the Schechter-Haller procedure for DDT and therefore is being described here. The sulfide is oxidized to the sulfone with hydrogen peroxide in acetic acid. The sulfone is nitrated, and treatment with sodium methylate produces a purple color (575 m$\mu$ maximum). Nitration of the sulfone yields a trinitro derivative rather than the tetranitro derivative as in DDT, but the position of these nitro groups has not been determined. Spray residues of Chlorbenside (98) may be slowly oxidized to either the sulfoxide or the sulfone and these will be reported as Chlorbenside using this method. However, both the biological and toxicological properties of these oxidation products are similar to those of Chlorbenside. The method is sensitive to 50 $\gamma$ and is relatively specific; however, it cannot be applied in the presence of DDT or related materials.

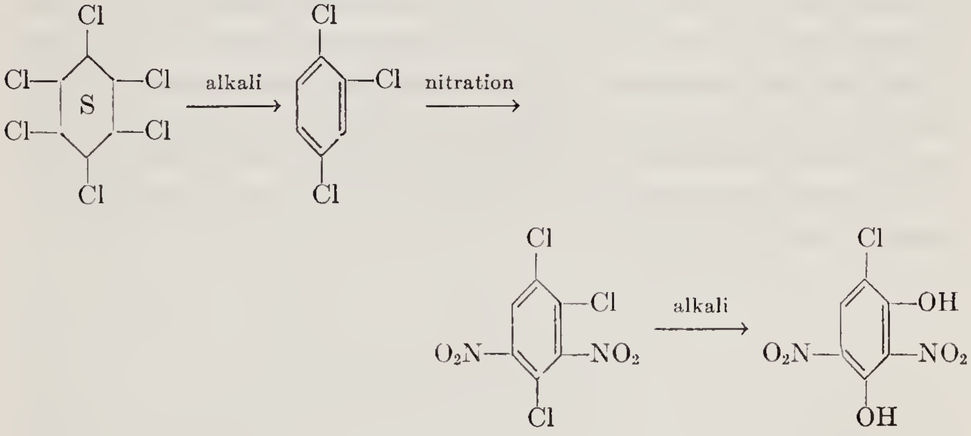
## D. BENZENE HEXACHLORIDE AND LINDANE

The chlorination of benzene can yield eight possible spatial isomers of 1,2,3,4,5,6-hexachlorocyclohexane. At least five of these isomers may be present in technical BHC but the gamma isomer is the only one of insecticidal value. The colorimetric methods available for microquantities of this insecticide ordinarily do not distinguish among the isomers. However, Klein (270) has devised a method for the beta isomer based on the colorimetric method of Schechter and Hornstein (392) and the observation (99) that the beta isomer is resistant to dehydrochlorination. In general, the methods described convert the hexachlorocyclohexane isomers to benzene or benzene derivatives.

Davidow and Woodard (111) have described an ultraviolet method. Alkaline hydrolysis of the BHC isomers yields a mixture of three trichlorobenzenes, with the 1,2,4-isomer being formed to the extent of about 80% regardless of the starting BHC isomer. The 1,2,4-

trichlorobenzene has an absorption spectrum in the ultraviolet region characteristically different from the other trichlorobenzenes and its absorbance is measured at 286 m$\mu$. It was found that the unsaponifiable fractions of many materials absorbed in the same region but the absorbance for these interferences was relatively linear from 284 to 290 m$\mu$. Two methods for elimination of this interference were employed. One depended on measurement of the absorbance at two wavelengths (284 and 286 m$\mu$) and application of simultaneous equations for the resolution of a two-component color system. The second method is a graphical method, in which a base line is ruled between absorbances at 284 and 290 m$\mu$. The base line absorbance at 286 m$\mu$ is directly proportional to the BHC concentration and is used for the preparation of the working concentration curve and for samples. The lower limit for this method is about 100 $\gamma$. Recovery data revealed standard deviations of ±8%.

Armstrong *et al.* (16) have described a colorimetric method developed by Southern and Williams for BHC. In this procedure the BHC isomers are dehydrochlorinated to trichlorobenzenes as in the Davidow-Woodard method (111). The trichlorobenzenes are nitrated and refluxed with alkali. The chemistry of these reactions has been elucidated by Bradbury and Standen (52) (VII). The alkaline

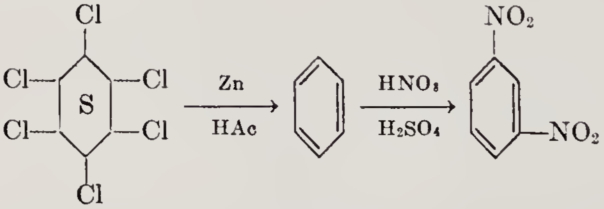


(VII)

hydrolysis of BHC isomers yields about 80% of 1,2,4-trichlorobenzene (plus other isomers). Nitration yields 1,2,4-trichloro-3,5-dinitrobenzene. The action of hot aqueous alkali produces a main product which is 6-chloro-2,4-dinitroresorcinol. The yellow color of the substituted resorcinol is read at about 415 m$\mu$. The other chlorodinitroresorcinols produced in small amounts show little absorbance at this wavelength. The losses at each stage were determined and losses during nitration and in the subsequent hydrolysis amounted to about 40% of the BHC originally present. However, results were reproducible.

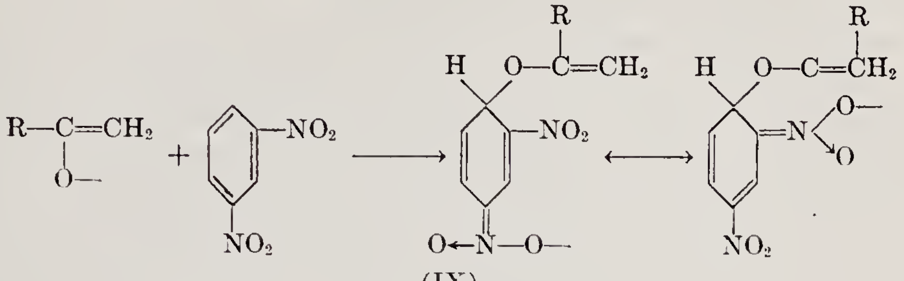
Schechter and Hornstein (392) have described a method based on the dechlorination of benzene hexachloride to benzene by means of zinc in acetic acid. The benzene is absorbed in a nitrating mixture and converted to *m*-dinitrobenzene, which after extraction is reacted with methyl ethyl ketone in the presence of strong alkali to yield a violet-red color measured at 565 mμ. The dechlorination and subsequent nitration are carried out in a specially designed all-glass apparatus. This method has its own "built-in" cleanup procedure since the benzene is distilled out of the reaction flask and is thus separated from interferences. Fats and other glycerides do not interfere and many materials can be analyzed directly without preliminary cleanup. Not all of the isomers are dechlorinated to the same extent in the time used for an analysis. The alpha, gamma, and epsilon isomers dechlorinate about 100%, the beta isomer 80–90%, and the delta isomer 30%. However, the delta isomer accounts for only about 5–10% of technical BHC. Technical BHC gives about 90% of the color given by the gamma isomer. The conversion of benzene to *m*-dinitrobenzene is not quantitative (any *o*- and *p*-isomers formed do not contribute to color formation); however, the conversion (about 85%) is consistent, and standard working curves both for lindane (99% gamma isomer or better) and for technical BHC follow Beers' law.

The reactions through the nitration step may be formulated as shown in equation (VIII).



(VIII)

The reaction between nitro compounds and ketones in the presence of concentrated alkali (the Janovsky reaction) has been extensively studied. Baernstein (24) summarized these studies and indicated that a condensation to form a quinoid structure takes place between the enol form of the ketone and the aci-nitro form of the *m*-dinitro compound (IX).


(IX)

As little as 5 γ of benzene hexachloride can be determined in this manner. A series of analyses at the 75 γ level gave an average deviation of ±0.5 γ. Although the method seems to be reasonably specific with regard to any significant interference from the commonly employed insecticides, any material that can be reduced or dehalogenated to benzene under the conditions employed would give the same color. In addition, certain aromatic compounds (396), if originally present or eventually produced, might also interfere. In using this method of analysis, benzene contamination must be scrupulously avoided. Zeumer and Neuhaus (462), Reith (376), Hancock and Laws (206), and Spillane and Pearce (413,437) have all described modifications of the apparatus and method. Gehrke and Bevirt (174) discussed some of the critical points in the method and found that the method as applied to lindane in pickles was subject to serious interferences. Hornstein (238) suggested a preliminary cleanup by sulfuric acid extraction for lindane in mushrooms, while Klein (270) removed interfering substances from peanut oil on Celite-fuming sulfuric acid. Hornstein and Sullivan (240) have determined lindane in air. Methods have also been described for BHC in peanuts and soils (237).

Davidow and Frawley (109) have shown that the beta isomer is preferentially stored in animal fat and the determination of this isomer is therefore desirable. Klein (270) has taken advantage of Cristol's observations (99) that the beta isomer is relatively resistant to alkali, and has established a set of dehydrohalogenation conditions in which the isomers other than beta are essentially all hydrolyzed to trichlorobenzenes while the beta isomer remains essentially intact. Trichlorobenzenes do not react with zinc in acetic acid since aromatic halides are inert to this treatment while the beta isomer does dechlorinate to benzene and its presence can therefore readily be detected by the colorimetric method (270).

Phillips (362) described an empirical method that depends upon refluxing BHC with aniline to give a mixture believed to contain among other products diphenylamine and at least two dichlorodiphenylamines. When microgram amounts of BHC are used, and the reaction products are oxidized with sulfuric acid-vanadium pentoxide, a red color is formed with an absorption maximum at 510 $m\mu$. The three components believed to contribute to the color formation have absorption maxima at 510, 555, and 595 $m\mu$. Kolthoff *et al.* (274) have shown that the oxidation of diphenylamine can produce a blue quinoid imonium salt, and it is believed that the colored products from the dichlorodiphenylamines are of this type. Beer's law is followed in the range of 2 to 120 $\gamma$, but the BHC isomers yield different amounts of color. The epsilon isomer produces 110%, alpha 90%, delta 40%, and beta isomer approximately 5% of the amount of the color given by the gamma isomer. Fats and other glycerides interfere and must be removed. This may be accomplished by Davidow's method (107).

Weber (444) converted BHC to nitrobenzene which was in turn reduced to aniline and determined.

## E. CHLORDANE AND HEPTACHLOR

### 1. *Chlordane (1,2,4,5,6,7,8,8-Octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene)*

The technical material contains alpha- and beta-chlordane and smaller amounts of heptachlor and a number of other compounds.

In general the methods are based on the reaction of the insecticide with strong alkali in the presence of an organic base. Ard (15) reported that chlordane when heated with pyridine, alcoholic alkali and ethylene glycol monoethyl ether gave an intense red color which was measured at a suitable wavelength in the region 460–530 $m\mu$. Palumbio (353) used alcoholic potassium hydroxide and *p*-aminophenol to develop a color. Davidow (108) increased the sensitivity and specificity of these methods by heating chlordane with diethanolamine in alcoholic potassium hydroxide and measured the color at 521 $m\mu$. The wavelength was chosen for making absorbance measurements where the absorption curves for equimolecular amounts of the colored reaction products obtained from the several chlordane constituents tended to intersect. In this manner the absorbance measurements are relatively uniform for a given chlordane concentration even though the composition may vary from batch to batch. The recovery of technical chlordane added to extracts of cabbage, pears, or biological tissue was 89% with an average deviation of ±5%. As little as 50 $\gamma$ of technical chlordane could be determined. Harris (209,210) described a method based on heating chlordane with methanolic potassium hydroxide, pyridine, and naphthalene. A violet color is obtained with an absorption maximum at 404 m$\mu$. Methylated naphthalenes and toxaphene interfere.

Ordas *et al.* (351) developed special chromatographic cleanup procedures for chlordane and heptachlor analyses.

### *2. Heptachlor (1 (or 3a),4,5,6,7,8,8-Heptachloro-3a,4,7,7a-tetrahydro-4,7-methanoindene)*

Polen and Silverman (365) have described a method in which heptachlor is heated with ethanolamine and potassium hydroxide in Butyl Cellosolve. The absorbance was measured in 95% ethanol at or near 564 m$\mu$. Heptachlor can be determined with a mean relative error of ±2% and as little as 5 $\gamma$ can be detected. Other common insecticides, with the exception of technical chlordane, do not interfere. The reagent is unstable and new calibrations must be made for each batch.

Heptachlor epoxide is a metabolite of heptachlor. Radomski and Davidow (372) used the same reagents and conditions employed for chlordane (111) in the development of a method for heptachlor epoxide. Heptachlor epoxide yielded a color, the absorption curve of which showed two maxima, one at 285 m$\mu$ and another about one-third as strong at 415 m$\mu$. The latter peak was less subject to background interference and was used to prepare the working curve. Although heptachlor will also give a color, there is no interference because of the wide difference in wavelengths of the absorption peaks.

## F. POLYCHLORINATED DIMETHANONAPHTHALENES

This series of insecticides is composed of aldrin, dieldrin, isodrin, and endrin. Aldrin and isodrin are geometric isomers, as are dieldrin and endrin.

Aldrin contains not less than 95% of 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-5,8-dimethanonaphthalene.

Dieldrin contains not less than 85% of 1,2,3,4,10,10-hexachloro-exo - 6,7 - epoxy - 1,4,4a,5,6,7,8,8a - octahydro - 1,4 - endo - exo - 5,8-dimethanonaphthalene.

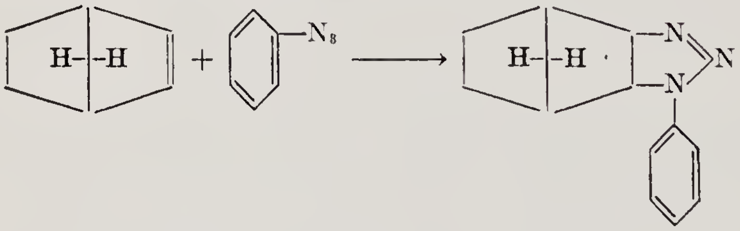
Isodrin is 1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-endo-5,8-dimethanonaphthalene.

Endrin is 1,2,3,4,10,10-hexachloro-exo-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-endo-endo-5,8-dimethanonaphthalene.

### *1. Phenyl Azide Method for Aldrin*

Danish and Lidov (104,105) described a colorimetric method for determining microgram quantities of aldrin and O'Donnell *et al.* (347) described an improved modification of this method. The reactions involved are as follows:

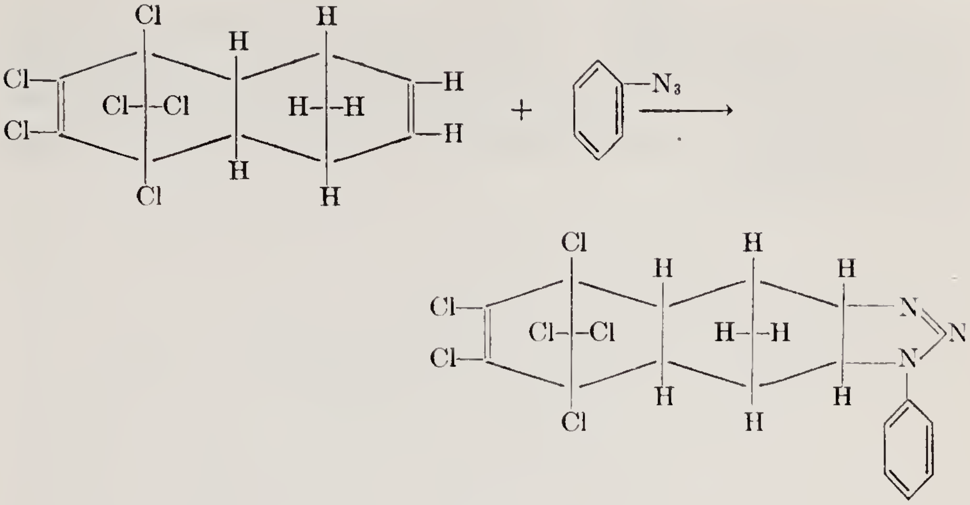
**(a) The addition of phenyl azide to aldrin.** This addition lends a substantial degree of specificity to the procedure since phenyl azide does not add to all double bonds. Alder and Stein (2) studied the addition compound formed between dicyclopentadiene and phenyl azide and found that this addition could be quantitative and proceeded readily with bicycloheptenes that contained a single carbon atom bridge as shown below. Thus, bicyclo [2.2.1] hept-2-ene and phenyl azide react readily to form a dihydrotriazole. The reaction may be formulated as shown in equation (X).



(X)

Aldrin, which contains the bicycloheptene structure, undergoes the same reaction (XI).

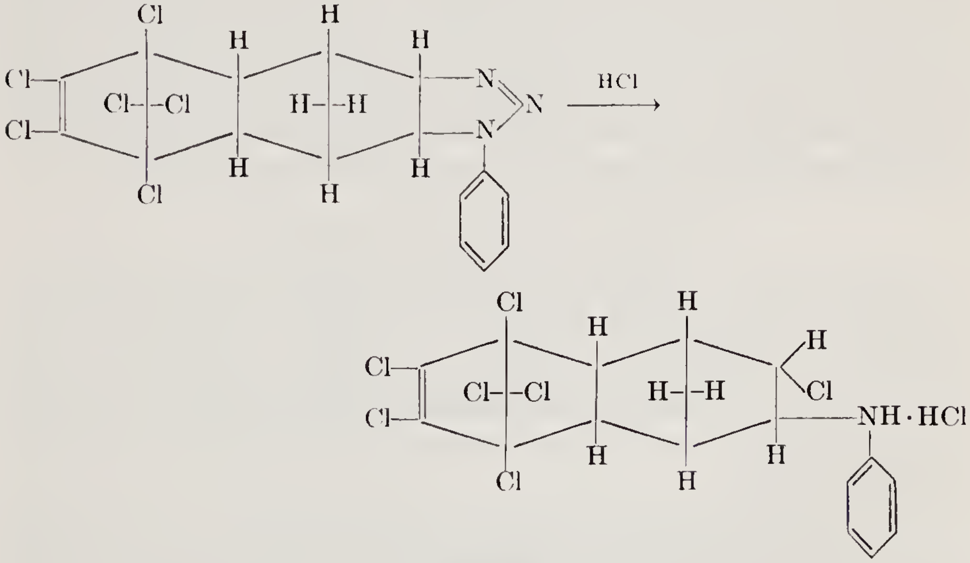
Since this addition can take place with terpenes that have this same bicycloheptene ring, cleanup procedures for plant extracts must be designed so that such materials are completely removed. In the evaluation of interferences from other insecticides, Danish and Lidov (104) found that other chlorinated insecticides did not interfere but



(XI)

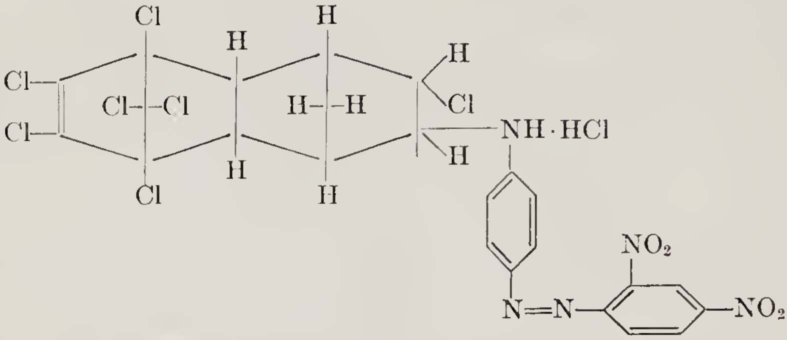
that Dimalone, a repellent, and MGK 264, a pyrethrum synergist, did interfere since a bicycloheptene ring is present in both compounds.

**(b) The dye-forming step.** In hydrochloric acid the aldrin-phenyldihydrotriazole decomposes to form a substituted aromatic amine (XII).



(XII)

Diazonium compounds readily couple in the *para* position with aromatic amines to give azo dyes. The amine is coupled with diazotized 2,4-dinitroaniline. The azo dye formed has been postulated as shown in formula (XIII). The addition of sulfuric acid converts this orange dye to a red dye the nature of which is not known and whose absorbance is measured at 515 mμ. Ten micrograms of aldrin can be determined.



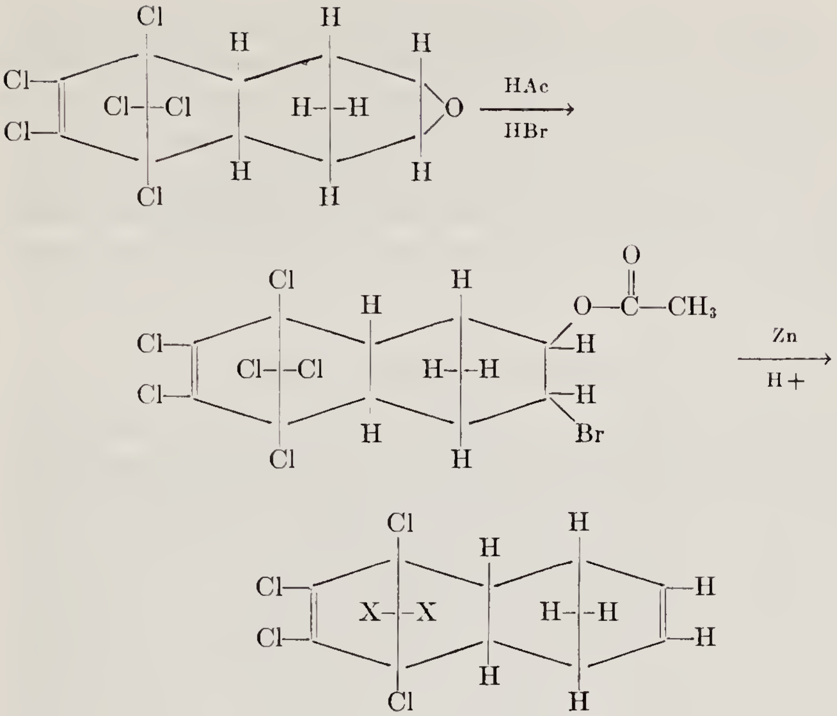
(XIII)

Since the color-producing reaction is a rather general one for aromatic amines and phenols, it is essential that even traces of such compounds be eliminated before the azo dye formation takes place. Care must also be taken that any excess phenyl azide is removed since its presence will give high results. The phenyl azide should be free from decomposition products since any amines formed would couple with the diazotized dinitroaniline to introduce interfering colors It is of interest to note that isodrin, the spatial isomer of aldrin, although it forms the triazole, will not couple with the diazotized dinitroaniline (347) (however, see the method for isodrin below).

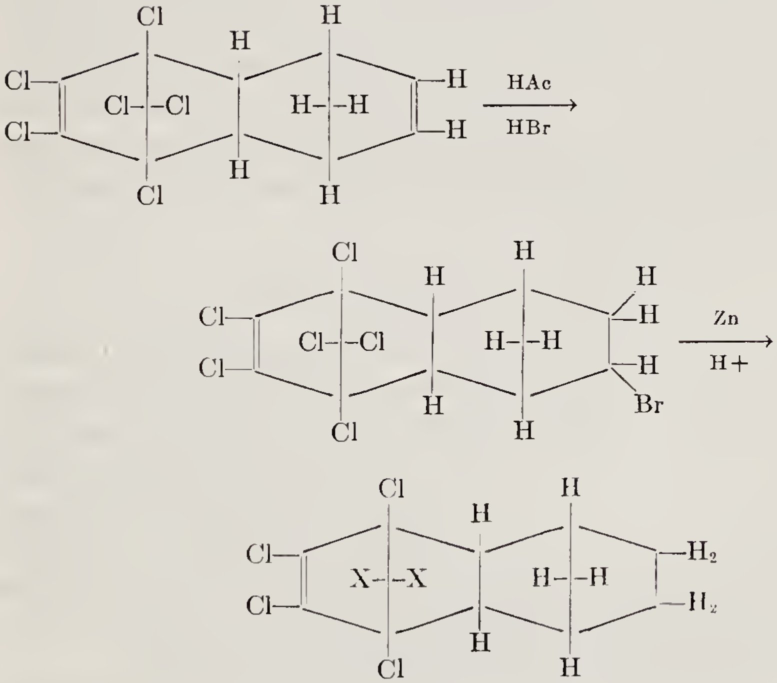
### 2. Phenyl Azide Method for Dieldrin

O'Donnell *et al.* (346) have applied the phenyl azide procedure to microgram quantities of dieldrin. In dieldrin an oxirane group (epoxy group) replaces a double bond present in aldrin. In this procedure, the oxirane ring is opened by heating dieldrin with hydrobromic acid-acetic acid to form 6-acetoxy-7-bromo-6,7-dihydroaldrin, equation (XIV).

Aldrin if present presumably reacts to add HBr (XV). Zinc dust is then added and the solution refluxed. The bromoacetoxy compound derived from dieldrin forms the olefin while the hydrogen bromide-addition product of aldrin can only form the dihydro derivative. The zinc reduction may remove the chlorine atoms on the methano



X = Cl or H (XIV)



X = Cl or H (XV)

bridge, but the vinyl- and bridgehead-chlorine atoms should be stable to this treatment. The converted dieldrin is then reacted with phenyl azide and carried through the remainder of the procedure used for aldrin. Toxaphene, which was reported not to interfere in the procedure for aldrin, does interfere when carried through this procedure for dieldrin. Toxaphene is a chlorinated camphene and zinc reduction probably introduces a bicycloheptene ring that can couple with phenyl azide.

### *3. Phenyl Azide Method for Endrin*

The method for dieldrin if applied to endrin would replace the epoxy group by a double bond, however, the spatial arrangement of the resulting compound would resemble isodrin which does not give the aldrin test. A recent bulletin (399) describes a modification that makes the phenyl azide method applicable to endrin. An ethanolic solution of endrin is heated with metallic sodium. The chlorinated bicycloheptene ring is dechlorinated by this treatment, the product is reacted with phenyl azide, and the addition product is coupled with diazotized sulfanilic acid. Treatment with sulfuric acid gives a red dye whose absorbance is measured at 515 m$\mu$. The method is claimed to be accurate for endrin concentrations as low as 0.05 p.p.m. with a minimum detection level of 5 $\gamma$. As might be expected, dieldrin interferes in this reaction. Technical chlordane, whose isomers also possess a chlorinated bicycloheptene nucleus, also interferes after sodium reduction, as does toxaphene. Surprisingly, aldrin is reported not to interfere after reduction.

### *4. Phenyl Azide Method for Isodrin*

The Shell Development Company has reported (400) that isodrin can also be determined by the original phenyl azide method. Isodrin-phenyldihydrotriazole is formed as for aldrin, and after removal of excess phenyl azide, 1-*o*-toluidine-2-propanol (not a diazonium salt) is added. To this solution a small amount of 60% perchloric acid is added followed by a trace of 90% hydrogen peroxide. A blue color forms on standing and the absorbance is measured at 590 m$\mu$. This method is reported to be accurate for as little as 0.1 $\gamma$ of isodrin and has been applied at the 0.1 p.p.m. level to a number of crops.

Garhardt *et al.* (173) described one of the few applications of infrared spectroscopy to residue methods. A sensitivity of 1 to 2 γ of aldrin or dieldrin was attained. Absorption microcells were used.

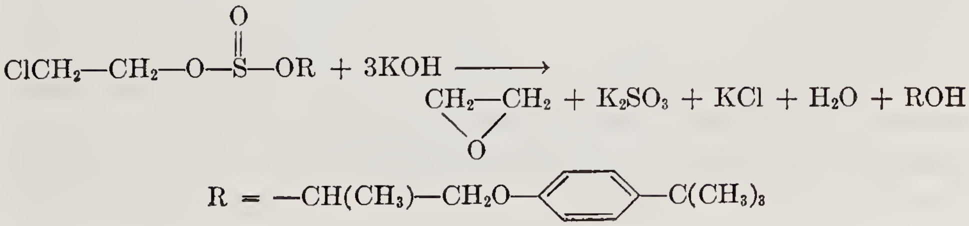
## G. TOXAPHENE AND STROBANE

Toxaphene and Strobane are closely related and are complex mixtures. Toxaphene is a chlorinated camphene having a chlorine content of 67–69%, and Strobane is a chlorinated terpene with a chlorine content of approximately 66%. The chemical structure of none of the components has been elucidated. Analytical methods generally depend on total chlorine determinations. Kenyon (263) suggested the use of the infrared spectrum for identification and used this as a qualitative procedure for toxaphene in fat. Johnson (254) described a rapid test for the detection of toxaphene in agricultural formulations based on heating toxaphene, pyridine, and methanolic potassium hydroxide (Fujiwara reaction). The optimum concentration was 2 to 6 mg. of toxaphene per milliliter. Hornstein (239) suggested a procedure in which toxaphene is heated with thiourea in the presence of potassium hydroxide in aqueous isopropanol to give a yellow color. Although the absorption peak is at about 315 mμ, readings are made at 400 mμ to minimize background interference. A minimum of about 0.5 mg. of toxaphene in 5 ml. of solution can be detected. The method is also applicable to Strobane. Chlordane and heptachlor interfere.

## H. CHLORINE-CONTAINING ACARICIDES

### 1. *Aramite [2-(p-tert-Butylphenoxy)isopropyl-2-chloroethyl Sulfite]*

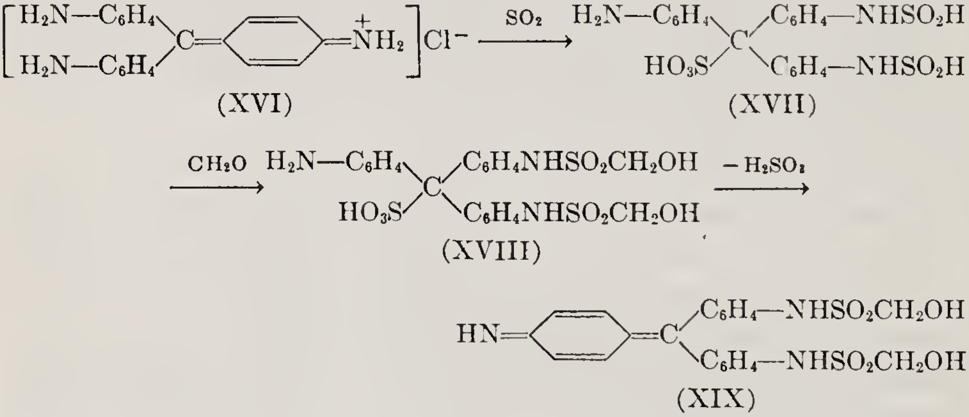
Gunther *et al.* (199,438) have described a method of analysis based on the quantitative liberation of ethylene oxide when Aramite is refluxed with potassium hydroxide and sodium isopropoxide in isopropanol. The ethylene oxide liberated is absorbed in a diethylene glycol solution of lepidine (4-methylquinoline). When heated to 170° an intense blue dye with an absorption peak at 610 mμ is formed. The reactions involved are:



$$\mathrm{ClCH_2{-}CH_2{-}O{-}\overset{\overset{O}{\|}}{S}{-}OR + 3KOH \longrightarrow \underset{\diagdown\, O\, \diagup}{CH_2{-}CH_2} + K_2SO_3 + KCl + H_2O + ROH}$$

The ethylene oxide probably reacts with lepidine to form a blue cyanine dye. The method is sensitive to about 5 micrograms. Cyclic oxides, imines, or sulfides if present or formed during the hydrolysis can interfere; however, their presence in an ordinary extract is problematical. Brokke *et al.* (54) have modified this procedure by hydrolysis of ethylene oxide to the glycol and oxidation of the latter with periodic acid to formaldehyde which is determined by the phenylhydrazine method.

Wood (456) has also described a method for Aramite based on the liberation of sulfur dioxide by hydrolysis with a methanol solution of sodium hydroxide. The sodium sulfite is determined colorimetrically by means of a fuchsin-formaldehyde reagent. The test is based on the fact that the dye resulting from the action of formaldehyde on fuchsin-sulfurous acid is more stable to strong mineral acid than fuchsin itself. The reagent contains fuchsin and a small amount of formaldehyde in acid solution. The addition of sulfite converts the fuchsin to fuchsin-sulfurous acid which then gives a red color with the formaldehyde already present. The reactions involved may be described as follows (401):



Sulfur dioxide reacts with fuchsin (XVI) to give a bis-*N*-aminosulfinic acid (XVII) which then combines with 2 moles of formaldehyde to produce the compound (XVIII); this is unstable and loses sulfurous acid to produce a quinoid dye (XIX). On standing the red dye slowly decomposes into formaldehyde-bisulfite and fuchsin-sulfurous acid so that the color is read at 580 mμ immediately after a fixed time interval. The method is sensitive to about 1 part of $SO_2$ in 500,000 parts of water.

### *2. Ovex (p-Chlorophenyl p-Chlorobenzenesulfonate)*

Kutschinski and Luce (279) developed an analytical procedure in which ovex (Ovotran) is quantitatively hydrolyzed to *p*-chlorophenol and *p*-chlorobenzenesulfonate by means of ethanolic alcoholic potassium hydroxide. The *p*-chlorophenol is then determined by an indophenol procedure described by Emerson (137) and improved by Gottlieb and Marsh (186). Gunther and Jeppson (200) applied this procedure to ovex residues in lemons and oranges and measured the absorbance of the color at 510 m$\mu$. They found the method capable of detecting 10 $\gamma$ within ±20%. Quinones, phenols, and phenolic esters may cause interferences.

### *3. Compound 923 (2,4-Dichlorophenyl Benzenesulfonate)*

2,4-Dichlorophenyl benzenesulfonate is an isomer of ovex and may be determined similarly by the method of Kutschinski and Luce (279).

### *4. Neotran [bis(p-Chlorophenoxy)methane]*

The analytical method is very similar to that for Ovex (279). However, the ether linkage is alkali stable and hydrolysis is carried out in 48% hydrobromic acid. The *p*-chlorophenol is steam-distilled and determined colorimetrically (120,279).

### *5. Sulphenone (R 242)*

Sulphenone usually consists of 80% of 4-chlorophenyl phenyl sulfone, 15% of diphenyl sulfone, and 5% of bis(4-chlorophenyl) sulfone. Patchett (356) has described a method for Sulphenone in plant and animal tissues based on a chromatographic separation of the Sulphenone followed by spectrophotometric analysis in the ultraviolet region. Quantities between 0.1 and 1.0 mg. are most conveniently determined and the minimum that can be detected is about 40 $\gamma$. Details of the cleanup and separation procedure are given in the section on cleanup procedures.

## VII. Organophosphorous Insecticides and Acaricides

Only chemical methods and a few enzymatic methods will be described in this section. General enzymatic methods have been described above.

### *1. Parathion and Methyl Parathion*

The most widely used method is that of Averell and Norris (22) or its modifications. The basic steps are extraction of the plant material with benzene, chromatographic cleanup on Attapulgus clay-Hyflo Supercel, evaporation of the benzene, reduction with zinc in acid solution to the amino compound, diazotization of the amino compound, and coupling with *N*-1-naphthylethylenediamine to produce a magenta color which is measured spectrophotometrically at 555 mμ. Amounts of 20–200 γ in the final 50 ml. aliquot are readily determined. The colored dye formed in the analysis has been identified as *N*-[4-*O*-*p*-phenylazo-(*O*,*O*-diethyl thiophosphate)-1-naphthyl]-ethylenediamine (47).

Gunther and Blinn (195) have adapted this method to a mass-production scheme for efficiently handling large numbers of analyses. Such factors as the preparation of samples, size of samples and subsamples, choice of solvent and its purification, extraction technique, storage of extracts, the effect of interferences and improvements of the analytical procedure have been discussed in considerable detail. Gunther and Blinn (195) and Edwards (127) have pointed out that traces of aniline or nitrobenzene in the solvents employed could interfere. Methyl anthranilate, which occurs naturally in grapes and citrus fruits, can interfere unless removed by a few acid washes of the benzene strip solution (45). Wilson *et al.* (451) have suggested some improvements in the Averell-Norris method such as using a tall flask and increased amounts of zinc to avoid losses of parathion before reduction. Edwards and Hall (130) and Williams (448) have described the purification of parathion for use as a standard for analytical work.

Clifford (91) and Edwards (128) reported that the Averell-Norris method, although the method of choice for residues, suffers from non-specificity. EPN, Chlorthion, methyl parathion, para-oxon, some impurities in parathion, chloronitrobenzenes, and other aromatic nitro compounds interfere, and certain untreated vegetables and tobacco give pink colors. Fahey *et al.* (143) in a study of the persistence of residues on apples and peaches have actually applied the Averell-Norris procedure to determine not only parathion but also methyl parathion and EPN. In contrast to the findings of Gunther and Blinn (195), Clifford found that benzene strip solutions when kept cold did not lose parathion on storage (91).

Gunther and Blinn (196) have adapted the Averell-Norris method as a quick qualitative test for parathion in citrus oils which can detect 25 p.p.m. of the insecticide. Ramos *et al.* (375) extracted parathion with acetonitrile from a hexane solution of olive oil and then utilized the Averell-Norris method with about 75% recoveries.

Zeumer and Fischer (461) have described a reduction and diazotization procedure basically similar to that of Averell and Norris except that urea is used to destroy excess sodium nitrite and alpha-naphthylamine is used as the coupling reagent instead of naphthylethylenediamine. The method is claimed to be somewhat more sensitive than that of Averell and Norris. Three to 5 $\gamma$ of parathion can be detected.

Gage (166) modified the method by employing reduction with zinc in toluene-acetic acid and extraction of the amine with dilute hydrochloric acid to avoid interference from plant pigments. The amine is diazotized and coupled with *N*-beta-sulfatoethyl-*m*-toluidine and the color measured at 510 m$\mu$. Gage (167) modified this procedure to enable the determination of parathion and such impurities as *p*-nitrophenol and the *S*-ethyl isomer of parathion. After reduction of the sample with zinc dust in toluene, the *p*-aminophenol produced from any *p*-nitrophenol which is present is determined as an indophenol dye by coupling with *o*-cresol. The reduced *S*-ethyl isomer of parathion is also determined as an indophenol dye after hydrolysis with alkali to *p*-aminophenol; the reduced parathion which is not hydrolyzed readily by alkali under the specified conditions can be determined by diazotization and coupling with *N*-sulfato-ethyl-*m*-toluidine.

Ketelaar and Hellingmann (265) described a method for the analysis of parathion and its formulations based on the colorimetric determination of *p*-nitrophenol after alkaline hydrolysis. Buckley and Colthurst (56) utilized a similar method for parathion residues on tomatoes in which plant pigments are oxidized with hydrogen peroxide. This method can detect about 0.2 p.p.m.

Biggs (38) utilized the ultraviolet absorption peak of parathion at 268 m$\mu$ for its determination especially in hexane extracts of viscera. Confirmation was attained by hydrolysis to *p*-nitrophenol and its measurement at 408 m$\mu$. Derkosch and Mayer (114) also used ultraviolet absorption for the detection and determination of parathion for forensic purposes. Hirt and Gisclard (233) and Carman *et al.* (66) have employed an ultraviolet method to determine parathion in air.

Methyl parathion may be determined by the same procedures used for parathion.

### *2. Chlorthion*

Kolbezen and Barkley (273) have modified the Averell-Norris parathion method to adapt it to Chlorthion residues. A longer reduction period and greater acidity in the final colored solution were found to be necessary. For milk analyses modifications to decrease difficulties due to the fat were incorporated. Concentrations greater than 0.1 p.p.m. could be determined spectrophotometrically (at 545 m$\mu$) with recoveries of 50–60%. As little as 0.02 p.p.m. could be approximated with Nessler tubes. No Chlorthion could be detected in milk samples from treated dairies. Fahey *et al.* (143) and the Chemagro Corporation (83) have also adapted the Averell-Norris method to Chlorthion residues.

### *3. EPN (O-Ethyl O-p-Nitrophenyl Benzenethiophosphonate)*

EPN residues may be determined (131) by the Averell-Norris method (22) which had been developed for parathion. The method is sensitive to 10 $\gamma$ of EPN with 95% recoveries. A method based on oxidation of the sample with a $HNO_3$-$KClO_3$ mixture and determination of the phosphorus by the molybdenum-blue reaction can also be used (131) for amounts down to 10$\gamma$ with recoveries of 95%. Neither method will distinguish between EPN and parathion, and the total phosphorus method is interfered with by other organophosphorus insecticides.

### *4. American Cyanamid 4124 [O,O-Dimethyl O-(2-Chloro-4-nitrophenyl) Thionophosphate]*

This insecticide has been determined (10) in milk by "freeze drying," extraction with benzene, and reduction with zinc to a water-soluble amino compound which is separated from the butter fat and determined by diazotization and coupling as in the Averell-Norris method (22) for parathion.

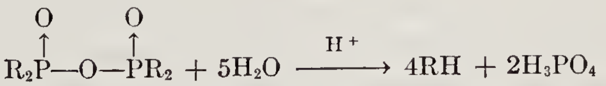
### *5. Schradan*

David *et al.* (106) have developed a method for schradan residues in which the macerated plant is extracted with water and the filtrate is hydrolyzed with 0.1 *N* alkali at 100° for 1 hour to remove lecithin and fatty phosphate esters (schradan is fairly resistant to alkaline hydrolysis). The schradan is extracted by chloroform from alkaline solution, the chloroform is evaporated, and the residue is hydrolyzed with dilute hydrochloric acid, treated with perchloric acid and evaporated to dryness. The phosphate is estimated colorimetrically by the molybdenum-blue method. A sensitivity of 0.1 mg. per kg. of plant tissue can be obtained in the absence of interference from natural compounds which may yield a blank value up to 1 mg. per kg.

Heath *et al.* (222) have applied a vacuum distillation to schradan residues. The plant is macerated and extracted with water, the water extract is made alkaline, extracted with chloroform and the solvent removed. The residue is subjected to a microdistillation at about 1 mm. with a bath temperature of 100°. The condensate on the cold-finger condenser contains the schradan which volatilized from the extract. The schradan is then determined by a molybdenum-blue method (35). Blanks are considerably lower than with David's method. Recoveries are reasonably good except for lemons, where the oil probably gives rise to difficulty. The validity of some of the steps involved was checked with radioactive schradan.

Casida *et al.* (78), after thorough study of the metabolism of schradan and the nature of the products formed in residues, suggested that residues be analyzed by a formaldehyde method (chromotropic acid-sulfuric acid) or an enzymatic assay procedure (cholinesterase or chymotrypsin) to determine the schradan metabolite, in conjunction with the method of David *et al.* (106) to determine unmetabolized schradan.

Hall *et al.* (201) developed a method for schradan residues based on chloroform extraction of the sample, hydrolysis to dimethylamine and phosphoric acid, steam distillation from excess alkali, absorption of the dimethylamine in dilute hydrochloric acid and development of a color with alkali, $Cu^{++}$, and $CS_2$ due to cupric dimethyldithiocarbamate (435 mμ maximum):



$$R_2\overset{\overset{O}{\uparrow}}{P}—O—\overset{\overset{O}{\uparrow}}{P}R_2 + 5H_2O \xrightarrow{H^+} 4RH + 2H_3PO_4$$

$$2RH + 2CS_2 + Cu^{++} + 2OH^- \longrightarrow Cu(—S—CS—R)_2 + 2H_2O$$

$$R = (CH_3)_2N—$$

The method is sensitive to about 5 γ and recoveries were about 93% on 25–100 γ amounts.

Ripper *et al.* (380) and Casida *et al.* (77) determined schradan residues by maceration of the plant in dilute alkali, heating to 80° for 30 minutes to hydrolyze lecithin-type compounds, and completion of the analysis as in the method of David *et al.* (106). The phosphate formed was determined by the molybdenum-blue method of Allen (7). Accurate results can be obtained on 0.1 mg. of phosphorus or 0.46 mg. of schradan and one hundredth of this quantity can be detected.

Although schradan is a weak cholinesterase inhibitor, its metabolic product, the *N*-oxide, is a strong inhibitor and a number of manometric enzyme inhibition methods have been employed (77,124,318, 319).

### *6. Dimefox (Hanane)*

Dimefox can be determined by the method of Dupée *et al.* (126). A filtered, aqueous macerate of the plant is made alkaline, extracted with chloroform, and the solvent evaporated. After addition of some glycerol and ethylene glycol as a carrier to aid in the distillation, the residue is distilled in a special microdistillation apparatus at 15 mm. and 180°. The condensate on the cold-finger condenser contains the dimefox which is then oxidized and the phosphate determined by a molybdenum-blue method (35). For oily crops and cocoa beans, the dimefox is distilled from the macerated plant material in the presence of a petroleum oil. The dimefox is then extracted into water, the solution is washed with petroleum-ether, and after the water solution is made alkaline, the dimefox is re-extracted into chloroform. It is then subjected to microdistillation after removal of the chloroform. Radioactive dimefox was used to check some of the steps in this method.

### *7. Demeton (Systox)*

No specific chemical procedures have been developed for this insecticide and only enzymatic methods are available. Hensel *et al.* (227) adapted the cholinesterase method of Giang and Hall (178) to the determination of demeton residues. By increasing the concentration of substrate, the time of hydrolysis, and the reaction temperature, the sensitivity was increased. About 0.1 $\gamma$ could be detected, or 0.1 to 0.2 p.p.m. with a 150-g. sample. Metcalf *et al.* (325) utilized the method of Hensel and also a manometric procedure and compared results with those obtained by radiometric methods. Paper chromatography was used to study the formation of different metabolites in the plant or by exposure to sunlight. Since the metabolites are more active cholinesterase inhibitors than demeton itself, they felt that a more realistic and accurate picture of toxic residues might be obtained by employing standard curves based on the more important metabolites—the thiolphosphate sulfoxide (five times as active), and thiolphosphate sulfone (ten times as active). The authors stated that the presence of these more toxic metabolites should offer no additional concern since the metabolism in animals (300) is essentially similar to that in plants. Furthermore, residues of demeton decrease rapidly to rather low values, usually less than 0.1 p.p.m. and the toxic metabolites readily hydrolyze to nontoxic derivates.

### *8. Diazinon*

Diazinon residues may be determined by the method of Suter *et al.* (426). The crop is stripped with benzene, the benzene solution is concentrated, petroleum ether is added, and the Diazinon is extracted into 48% hydrobromic acid. The acid solution is refluxed and the liberated hydrogen sulfide is swept into a zinc acetate absorbing solution and converted into methylene blue by means of *p*-aminodimethylaniline and ferric chloride. The color is read at 670 m$\mu$ and as little as 10 $\gamma$ of Diazinon may be determined. Sulfur and fungicides of the thiocarbamate type interfere. Recoveries were over 80% on residues from a number of crops. Where there are interferences in the hydrogen sulfide method, Diazinon residues may be determined by extraction with petroleum ether, washing to remove soluble phosphates, and evaporation. The dried residues are hydrolyzed by heating with hydrobromic acid and the phosphorus determined by the molybdenum-blue reaction. As little as 25 $\gamma$ of Diazinon can be determined with recoveries of over 88% (9). Other phosphates, arsenic, and silica interfere. Diazinon was also determined spectrophotometrically by the method of Harris (9,208) after hydrolysis to 2-isopropyl-4-methyl-6-hydroxypyrimidine which is separated from interfering substances and measured at 272 m$\mu$ in isopropanol. Recoveries range from 80 to 114% The authors' (426) analysis of residues by these three procedures shows good agreement above 1 p.p.m., fair agreement at 0.5–1 p.p.m. and greater divergence below 0.5 p.p.m. because of interferences. They have also used infrared absorption and turbidimetric tests with heteropoly acids to detect small amounts of Diazinon.

Blinn and Gunther (46) have improved the ultraviolet absorption procedure of Harris (208) for Diazinon. After a cleanup procedure, the Diazinon is hydrolyzed with alkali to 2-isopropyl-6-methyl-4-pyrimidinol. Extraneous matter is extractively removed from strongly acidic and basic aqueous solutions of the pyrimidinol which is eventually determined by its ultraviolet absorption at 272 m$\mu$ in ethanol. Recoveries of 63–70% are obtained.

Diazinon on apples has also been analyzed (9) by the enzymatic method of Cook (94).

### *9. DDVP (Dimethyl 2,2-Dichlorovinylphosphate)*

Giang *et al.* (179) have modified the enzymatic method of Giang and Hall (178) for DDVP on subtropical fruits by extraction of the DDVP with chloroform, evaporation of the extract in the presence of 20% aqueous alcohol, adjustment to pH 7, and measurement of enzyme inhibition. With this method, which is sensitive to 0.5$\gamma$, it was found that DDVP residues virtually disappeared after 72 hours.

### *10. Phosdrin (1-Carbomethoxy-1-propen-2-yl Dimethyl Phosphate)*

Casida *et al.* (79) determined residues of Phosdrin by a modification of the enzymatic method of Hensel *et al.* (227). It may be determined down to 0.05 p.p.m.

### *11. Dipterex (Bayer L 13/59)*

Giang *et al.* (177) have developed a sensitive procedure for Dipterex and some of its homologs by pyrolysis at 550° to liberate chloroform which is determined by a modified pyridine-alkali Fujiwara test. Although the method is sensitive to 20 $\gamma$, 1 p.p.m. in milk could not be determined because of interfering colors, and no attempts were made to apply the method to other residues. Chlordane, heptachlor, aldrin, dieldrin, endrin, and toxaphene gave some degree of interference.

Du Bois and Cotter (123) employed rat-brain cholinesterase and the manometric procedure of Dubois and Mangun (125) to determine Dipterex in milk and other foods. Dipterex could be determined directly in 1 ml. of milk down to 0.75 p.p.m. Quantities of Dipterex lower than this were determined after extraction of the pesticide with chloroform. The cholinesterase inhibition method of Hensel *et al.* (227) has also been used (82) for residues.

### *12. Isolan*

Suter *et al.* (426) determine Isolan by saponification and determination of the diethylamine by Dowden's procedure (121).

### *13. Malathion*

Norris, Vail, and Averell (342) described a sensitive colorimetric method for malathion based on extraction of the plant with carbon tetrachloride and decomposition of the insecticide in carbon tetrachloride–ethyl alcohol solution by alkali to give sodium dimethyl dithiophosphate and sodium fumarate. The sodium dimethyl dithiophosphate is extracted into water and converted into the copper complex of dimethyl dithiophosphoric acid which is then extracted into carbon tetrachloride and determined colorimetrically at 418 mμ. Blanks on untreated plants were usually less than 0.1 p.p.m. except for lettuce which gave a blank of about 0.3 p.p.m. Amounts of 0.25–2.5 mg. in 100 ml. aliquots of carbon tetrachloride extracts of plant material are readily determined in a wide variety of crops The method is not applicable to animal tissues (218). This method has been adapted to the analysis of milk (sensitivity about 100 $\gamma$ or about 0.5 p.p.m. in a 200 g. sample) but none could be detected in milk from cows grazing on malathion-treated pastures (180). Malathion residues decrease rapidly in a week or two (235,407,446) and can be reduced by washing (408).

### *14. Guthion (Bayer 17147)*

A specific test for Guthion has been developed by Wollenberg and Schrader (455) based on hydrolysis to a diazonium compound followed by coupling with phenyl alpha-naphthylamine to give a blue-violet color (570 mμ maximum); however, the method has not yet been applied to residues. Giang (176) reported that this compound can be determined by a modification of the Norris *et al.* method (342) for malathion; however, difficulty was experienced with actual residues, possibly because the compound may have been metabolized.

### *15. Trithion (Stauffer R-1303) S-(p-Chlorophenylthiomethyl O,O-Diethyl phosphorodithioate)*

A colorimetric method for the determination of Trithion residues has been developed (416). After extraction, chromatographic cleanup, and alkaline hydrolysis in ethylene glycol to *p*-chlorothiophenol, the latter is determined colorimetrically by the Gibbs method (140) by reaction with 2,6-dibromo-*N*-chloro-*p*-quinoneimide at pH 6.6 and measurement of the orange color at 480 mμ. The recoveries averaged 93% in the range of 0.05–0.40 p.p.m. with a standard deviation of ±7%. Of the commonly used pesticides, ovex (Ovotran) is the only one not removed by the chromatographic cleanup and interferes by causing a reduction in the amount of color produced.

### *16. Thimet (American Cyanamid 3911)*

Preliminary data (11) indicate that this compound is metabolized in a manner similar to demeton and that the metabolites are stronger ChE inhibitors than the original insecticides. As little as 0.005 p.p.m. of the metabolites may be detected by a ChE method that is being developed.

### *17. Antidotal Therapy*

All workers handling ChE inhibitors should be familiar with the antidotal therapy which consists of immediate massive doses of atropine (188,205).

## VIII. Nitrated Phenols, Cresols, and Other Aromatic Nitro Compounds

Avens *et al.* (21) determined dinitro-*o*-cresol by extraction from bark with petroleum ether, then extraction into sodium bicarbonate solution and colorimetric determination. Parker's method (355) for biological fluids depends on extraction from a sodium chloride–sodium carbonate solution with methyl ethyl ketone and measurement at 430 mμ for 1–10 γ/ml. amounts. Fenwick and Parker (151) have improved the method to remove interference from beta-carotene.

Davis and Selman (113) determined 2-*sec*-butyl-4,6-dinitrophenol by steam-distillation from soil after acidification with phosphoric acid. The distillate was collected in dilute sodium hydroxide solution and determined at 390 mμ.

Fischer (152) has extended the use of the Janovsky reaction (245, 246) to the determination of 1,3-dinitro-2,4,5-trichlorobenzene, 1,3,5-trinitro-2,4,6-trichlorobenzene, 2,4-dinitrothiocyanobenzene, 1,3,6,8-tetranitrocarbazole, and 1,8-dichloro-3,6-dinitrocarbazole.

Porter (369) has developed a color reaction for 5–100 γ of mononitro and some dinitro compounds with tetraethylammonium hydroxide in dimethylformamide; he also gives data on the Janovsky reaction for many mono and dinitro compounds as have Bost and Nicholson (50).

Auerbach (18) has described a method for tetrachloronitrobenzene by a color reaction (maximum at 548 mμ) in acetone with tetraethylammonium hydroxide. The method of Canbäck and Zajaczkowska (61) employing alkali, procaine hydrochloride, and alpha-naphthylamine as reagents may also be used (maximum at 500 mμ). Webster and Dawson (445) have determined this pesticide polarographically with residues as low as 0.05 p.p.m. and recoveries of 84–93%.

A method has been described (384) for Karathane [dinitro(1-methylheptyl) phenyl crotonate] in which a benzene extract is evaporated to dryness and then hydrolyzed with 1:1 sulfuric acid. The dinitroalkylphenol is steam-distilled, extracted into chloroform, the chloroform evaporated, and the absorbance of the dinitroalkylphenol is measured at 440 mμ in pyridine.

## IX. Herbicides

### *1. 2,4-Dichlorophenoxyacetic Acid (2,4-D) and 2,4,5-Trichlorophenoxyacetic Acid (2,4,5-T)*

Freed (157) described a qualitative test for 2,4-D (and certain other acids) employing chromotropic acid in sulfuric acid heated to about 150°. This test undoubtedly depends on the formation of formaldehyde from the herbicide. Marquardt and Luce (305) have adapted this test to the quantitative determination of 2,4-D in milk. They separate the 2,4-D by making use of its acidic properties and determine it with chromotropic acid–sulfuric acid by heating at 130–135°. The purple color is measured at 565 mμ. It is possible to detect 0.2 p.p.m. and probably less. These authors have extended this method to 2,4-D in grain and seed (306). They grind the grain or seed and extract with ether-acetic acid. Further treatment, including chromatography, removes interfering materials. The color produced with chromotropic acid in concentrated sulfuric acid is read at 565 mμ; the use of stannous chloride helps to reduce amber-colored interferences. Less than 0.05 p.p.m. of 2,4-D can be detected. Munakata (338) also developed a method for the determination of 2,4-D with chromotropic acid–sulfuric acid.

Bandurski (26) determined 2,4-D in soil leachates by means of the ultraviolet absorption at 284 mμ but interferences gave rise to difficulties. Warshowsky and Schantz (443) improved this procedure by means of a 24 plate countercurrent distribution between tributyl phosphate and a phosphate buffer of pH 7, and determination at 284 mμ. This method is involved and not adapted to routine residue studies; however, it illustrates what can be accomplished by the countercurrent technique by way of separation, purification, and identification of organic compounds used as pesticides and the possibilities of detecting metabolites and breakdown products.

Gordon and Beroza (185) have employed chromatography and ultraviolet absorption to determine small amounts of 2,4-D and 2,4,5-T but their method is not sensitive enough for most spray residue problems; neither is the chromatographic method of Stroud (423). However, these procedures might find application as a means of separating mixtures of herbicides or for cleanup of spray residues.

### *2. 2-Methyl-4-chlorophenoxyacetic Acid (MCP)*

Marquardt and Luce's method for 2,4-D has been adapted (333) to the determination of MCP by the use of silicic acid and 1% methanol in chloroform for the chromatographic step.

### *3. Crag Herbicide 1 (Sodium 2,4-Dichlorophenoxyethyl Sulfate), Natrin (Sodium 2,4,5-Trichlorophenoxyethyl Sulfate), and Related Compounds*

The determination (64) of sodium 2,4-dichlorophenoxyethyl sulfate and its hydrolysis products, 2,4-dichlorophenoxyethanol and 2,4-dichlorophenol, is an interesting example of the dye-complex method. In this method, sodium 2,4-dichlorophenoxyethyl sulfate and its hydrolysis products are extracted from the plant with water. Chloroform extraction of the solution removes the hydrolysis products. The herbicide remaining in the aqueous layer is determined by the addition of methylene blue chloride solution and extraction of the dye-complex into chloroform followed by spectrophotometric measurement at 650 mμ. The hydrolysis products, 2,4-dichlorophenoxyethanol and 2,4-dichlorophenol, in chloroform solution, are reacted with chlorosulfonic acid and the resulting sulfates are determined by the methylene blue complex method; both compounds have the same absorbances for the same amounts at low concentrations. The procedure is claimed to be applicable to solutions containing 3–220 γ of the herbicide and 4–320 γ of its hydrolysis products. The experimental herbicide sodium 2,4,5-trichlorophenoxyethyl sulfate (Natrin) can be determined similarly (63).

### *4. Trichloroacetic Acid (TCA)*

Barrons and Hummer (30) utilized the Fujiwara reaction to determine TCA. In alkaline solution it breaks down to chloroform which gives a magenta color with pyridine and alkali. As little as 1 γ of TCA can be detected.

Tibbitts and Holm (428) in a similar method, extracted TCA from plant tissue with 0.1 N acetic acid, filtered through asbestos to remove chlorophyll pigments, and filtered again after making the solution alkaline to remove interferences. The color was developed by pyridine and alkali and read at 510 mμ. The method has been applied to carrots, peas, snap-beans, millet, spinach, and beets. These authors determined as little as 25 γ of TCA per gram of fresh plant tissue.

Another method (119) for TCA (or its sodium salt) depends on extraction of the plant material with 5% acetic acid solution and boiling the solution to effect decarboxylation. The chloroform is swept into an absorber containing pyridine and alkali and determined colorimetrically (Fujiwara test) at 530 mμ. Amounts of TCA in the range 0.1–2.0 p.p.m. may be analyzed.

### *5. Isopropyl-N-phenylcarbamate (IPC)*

Bissinger and Fredenburg (40) determined IPC by extraction with methylene dichloride. evaporation, hydrolysis with a phosphoric acid-hydrochloric acid-acetic acid mixture, alkalinization, steam-distillation of the aniline, and colorimetric determination of the latter. As little as 0.33 p.p.m. of IPC was determined with a 99% recovery.

### *6. Isopropyl-N-(3-chlorophenyl)carbamate (CIPC)*

Gard and Rudd (168) have used a similar procedure for CIPC in soils, cotton seeds, and various horticultural crops. Dilute sulfuric acid (1:1) was used for hydrolysis to liberate 3-chloroaniline which was determined. As little as 0.05 p.p.m. of CIPC can be determined with a 200 g. sample. The average recovery was 89% and the average precision based on 95% confidence limits at the 0.05 p.p.m. level was ±0.016 p.p.m. The herbicide was extracted from cottonseed oil with acetonitrile prior to analysis. Gard *et al.* (169) have applied this method to lettuce, sugar beets, onions, cotton seeds, peanuts, and spinach which had received pre-emergence treatments of 2.5–8.0 lb. of CIPC per acre and found less than 0.05 p.p.m. in every case.

### *7. Naphthyl- and Tolyl-phthalamic Acids*

Smith and Stone (406) have determined residues of *N*-1-naphthylphthalamic acid (Alanap-1) by basic hydrolysis to yield 1-naphthylamine which is steam-distilled and determined colorimetrically after coupling with diazotized sulfanilic acid. Two micrograms of the herbicide may be detected and recoveries are 80–100% in the 5 $\gamma$ range. No residues were found in any of the crop samples analyzed. *N-m*-tolyl phthalamic acid may be determined similarly (422) by basic hydrolysis, steam distillation of the *m*-toluidine, diazotization, coupling with *N*-(1-naphthyl)ethylenediamine and measurement of the color at 560 m$\mu$.

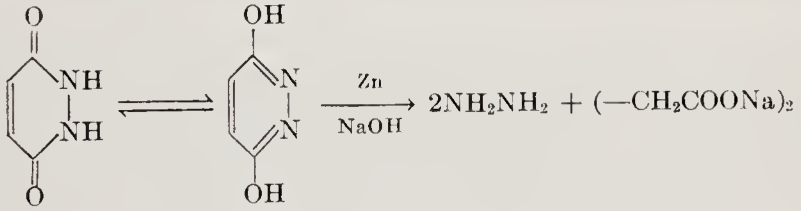
### *8. 3-(p-Chlorophenyl)-1,1-dimethylurea (CMU)*

Bleidner *et al.* (44) have developed a method for herbicidal residues of CMU by digestion in strong alkali to give *p*-chloroaniline which is determined, after extraction or steam distillation, by diazotization and coupling with *N*-(1-naphthyl)ethylenediamine and measurement at 560 m$\mu$. This method is a modification of the earlier procedures by Lowen and Baker (298) and Young and Gortner (460); the latter authors could determine 0.01 p.p.m. of CMU in 300 g. of pineapple samples and recoveries were 97–99%. Recoveries of 76–102% from soils and 83–108% from various crops were found. Standard deviations of 4.8% relative at a 1.23 p.p.m. level and 4.6% at a 2.36 p.p.m. level were attained. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea can be determined similarly.

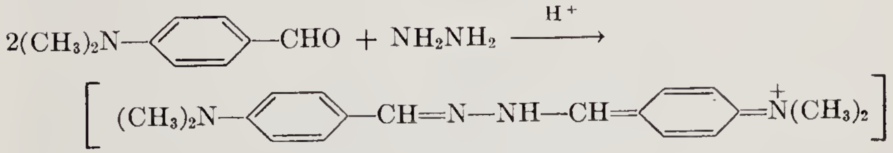
Bleidner (43) has improved his earlier method (44) by removal of interference due to *o*-aminoacetophenone which is produced in the course of hydrolysis of plant tissues (and of tryptophan). Bleidner demonstrated with radioactive CMU and paper chromatography that the *p*-chloroaniline formed by alkaline hydrolysis is a true index of the maximum herbicide residue. Incorporation of a chromatographic separation of the dyes resulting from *p*-chloroaniline and *o*-aminoacetophenone on a powdered cellulose column removed interferences and made the method more sensitive.

### *9. Maleic Hydrazide*

Maleic hydrazide can be determined in plant and animal tissues and soil by the method of Wood (457). It is stable to acidic and basic hydrolysis but will hydrolyze after reduction. Wood resorted to a combined reduction-hydrolysis procedure with zinc and alkali in water, followed by the colorimetric determination of the steam-distilled hydrazine by reaction with *p*-dimethylaminobenzaldehyde. A special reduction-distillation apparatus was used. The color was read at 455 mμ and recoveries were 90–100%, with duplicates usually checking to within 10% or better. Sulfur dioxide-liberating compounds as well as large amounts of copper interfere with color development. Only 0.5 to 3 g. of sample from a larger sample blend is required for analysis. The method is satisfactory at 0.5 p.p.m. and possibly down to about 0.25 p.p.m. The reactions are shown in equations (XX) and (XXI).



(XX)



(XXI)

### *10. 3-Amino-1,2,4-triazole (Aminotriazole)*

Sund (425) has developed a colorimetric method for aminotriazole residues in soil depending on the green color (634 mμ maximum) produced with sodium nitroprusside. Complicating factors in the analysis of this pesticide in soils are its strong adsorption, either as such, or as a heavy metal complex, especially in muck soils and those having high ion-exchange capacities. There is a strong inverse correlation between the percentage of original residue recovered and the base-exchange capacity of the soil.

#### *11. Dichloralurea (1,3-Bis(2,2,2-trichloro-1-hydroxyethyl)urea*

Residue methods for dichloralurea in sugar beets have been developed (65) which depend on the color produced (530 m$\mu$ maximum) with pyridine and alkali (Fujiwara test).

## X. Fungicides

### A. ORGANIC MERCURY COMPOUNDS

McCallan *et al.* (312) list approximately twenty-five organic mercury compounds that may be used as fungicides. In general the analysis of these compounds consists of a wet oxidation followed by formation of a mercury dithizonate whose absorbance is then measured (see section on inorganic pesticides). Reactions between intact mercury-organic compounds and dithizone have been made the basis of relatively specific methods.

#### *1. Diphenyl Mercury, Phenyl Mercuric, and Ethyl Mercuric Compounds*

Miller *et al.* (329) described a procedure using dithizone for these intact metallo-organics even in the presence of heavy metal ions including mercury. Metallic ions are extracted from the chloroform-dithizone solution by $3N$ hydrochloric acid. The phenyl- or ethyl-mercuric compound is retained in the organic phase. The metallo-organic concentration is determined by measurement of the unreacted dithizone at 620 m$\mu$. The method may be used to determine approximately 1–150 $\gamma$ of phenyl mercuric or ethyl mercuric compound. Polley and Miller (366) also described a method for mixed phenyl- and ethyl-mercuric compounds based on the observation that phenyl mercuric compounds break down in $12N$ HCl to give inorganic mercury while ethyl mercuric compounds are relatively stable. The procedure as described by Miller *et al.* (329) is used to determine the ethyl mercuric compound. The mercury obtained from the phenyl compound may be determined in the aqueous phase. The over-all accuracy is 3%. Miller and Polley (328) determined diphenyl mercury alone or in the presence of the phenyl mercuric compound.

Diphenyl mercury alone is determined by hydrolysis in $9N$ HCl to give phenyl mercuric chloride which is determined as in (329). If diphenyl mercury and phenyl mercuric compounds are present together, only the latter is determined in $0.05\ N$ acid since it alone reacts with dithizone at this acidity. The phenyl mercuric compounds can be extracted with acid thiosulfate (84) and the diphenyl mercury in the organic phase analyzed (328). The same procedures may be used to determine diethyl mercury and ethyl mercuric compounds.

### *2. Panogen (Methyl Mercuric Dicyandiamide)*

Polley and Miller (368) have described a direct method for this compound which depends on conversion of the extremely water-soluble fungicide to methyl mercuric chloride which partitions favorably into chloroform thus separating it from heavy metal ions. The customary dithizonate is then formed. As little as 2 $\gamma$ can be determined.

## B. ORGANIC COPPER COMPOUNDS

The copper content of organic copper compounds may be made the basis of their determination (see section on inorganic pesticides).

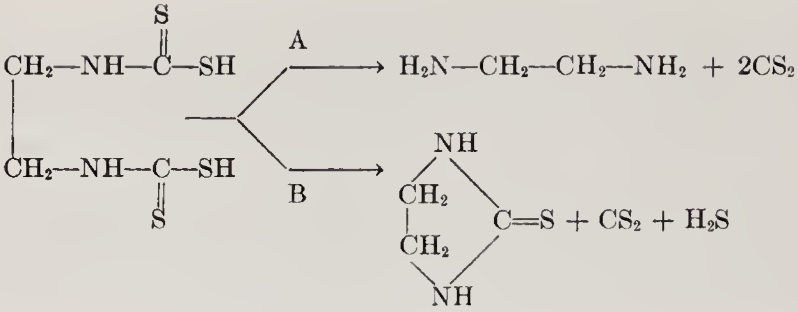
## C. ORGANIC SULFUR- OR NITROGEN-CONTAINING FUNGICIDES

### *1. Dithiocarbamates*

The compounds currently used are: (a) Ferbam (Fermate), ferric dimethyl dithiocarbamate; (b) Ziram (Zerlate), zinc dimethyl dithiocarbamate; (c) Maneb (Manzate), manganese ethylene bisdithiocarbamate; (d) Zineb (Dithane Z-78), zinc ethylene bisdithiocarbamate; and (e) Nabam (Dithane D-14), disodium ethylene bisdithiocarbamate.

Clarke *et al.* (88) developed a method for determining micro amounts of these compounds based on Callan and Strafford's (60) procedure of liberating carbon disulfide quantitatively by acid decomposition of the dithiocarbamate. The carbon disulfide, after the gas is bubbled through a lead acetate trap to remove any sulfide- or sulfur dioxide-producing impurities in the sample, is absorbed in a copper acetate–diethylamine solution to form cupric diethyl dithiocarbamate (430 m$\mu$ maximum) (115,439).

Bisdithiocarbamates can decompose in two different ways (88) (XXII). Reaction A is quantitative in dilute sulfuric acid at 100°



(XXII)

for nabam and zineb. Lowen (297), however, found that maneb tended to follow course B unless the maneb sample was dispersed in water-alcohol and heated for a longer period of time. Levitsky and Lowen (286) found that dispersions made with aqueous Versene could be satisfactorily analyzed.

Lowen (296) using Clarke's procedure (88) found that as little as 10$\gamma$ of a dithiocarbamate could be determined. Leafy vegetables required pulping of the sample with water in a homogenizer and decomposition of the dithiocarbamates directly in the aqueous suspension by the addition of acid. Recoveries, particularly for zineb, were poor. Appropriate factors, determined by the use of controls containing known amounts of fungicides, were used to correct for low recoveries of carbon disulfide. Rosenthal *et al.* (385) studied interferences in the analysis of zineb as applied to formulations and found that arsenates and copper salts tended to give low results. Substitution of 85% phosphoric acid for dilute sulfuric acid in the acid decomposition of the dithiocarbamate gave better recoveries.

### *2. Thiram (Tetramethylthiuram Disulfide)*

Thiram probably can be determined by alkaline reduction followed by hydrolysis to dimethyl dithiocarbamic acid and determination of the latter (308).

### *3. Captan (N-Trichloromethylmercapto-4-cyclohexane-1,2-dicarboximide; N-Trichloromethylthiotetrahydrophthalimide)*

Kittleson (267) has described a method that consists of heating resorcinol with the fungicide at 135–140° to form an intense red-colored compound. Although the structure of the reaction product has not been precisely determined, Kittleson found the absorption curve for the resorcinol-tetrahydrophthalic anhydride condensation product to be almost identical with the curve obtained by heating Captan and resorcinol and he concluded that the chromogenic reaction product was of the phthalein type. The absorbance was measured in ethanol at 425 mμ and as little as 50 γ could be determined. Petrino (360) and Martin and Pickard (310) have suggested the use of preliminary chromatography.

### *4. Glyodin (Crag 341; 2-Heptadecylglyoxalidine Acetate)*

Hillenbrand *et al.* (232) determined this compound by the dye-complex method employing methylene blue and chloroform. The method did not distinguish between the several related glyoxalidines present in the commercial product. A dye-complex method employing bromocresol purple has also been described (62).

### *5. B-622 (2,4-Dichloro-(6-o-chloroanilino) triazine)*

This compound may be determined directly (364) by heating with pyridine in benzene or water to form the quaternary ammonium salt which yields a yellow color on the addition of alkali. A second method (364) is to hydrolyze the triazine in acid to *o*-chloroaniline which is then diazotized and coupled with *N*-(1-naphthyl)ethylenediamine. The absorbance is measured at 545 mμ.

## D. PHENOLS AND QUINONES

### *1. Pentachlorophenol*

Pentachlorophenol may be oxidized in benzene solution with nitric acid–hydrochloric acid to tetrachloro-*o*- and tetrachloro-*p*-quinones. The color is determined at 450 mμ (332). Pentachlorophenol can also be determined colorimetrically as a copper-pyridine complex or by means of its ultraviolet absorption (332). Wallin (442) described a method based on the solubility of the copper salt in 70% isopropanol. The addition of potassium ferrocyanide to the isopropanol solution gives the copper ferrocyanide color (maximum at 415 mμ). Wallin (442) also described a dye-complex method based on the reaction of the phenol at pH 10.9 with methylene blue chloride. Haskins (217) showed that safranin-O in bicarbonate-buffered solution also forms chloroform-soluble dye-complexes with tetra- and penta-chlorophenols.

### *2. o-Phenylphenol (Phenylphenol)*

Tomkins and Isherwood (429) described a method for *o*-phenylphenol in citrus fruit peel. The phenol is steam-distilled and extracted from an acidified solution with petroleum ether. Alkali is used to extract the phenol from the organic phase. The phenol is then coupled with diazotized sulfanilic acid, and the color is matched with a standard.

### *3. Tetrachloro-p-benzoquinone (Chloranil, Spergon)*

Burchfield and McNew (57) describe a procedure based on the reaction of tetrachloro-*p*-benzoquinone with alkaline reagents to form highly colored substitution products. The fungicide is removed from the seed with acetone and the solution treated with diethylamine. The yellow color formed is measured at 450 m$\mu$.

### *4. Dichloro-1,4-naphthoquinone (Phygon)*

Burchfield and McNew (58) described a modification of their method for tetrachlorobenzoquinone (57) for this compound. The acetone extract was treated with 10% aqueous dimethylamine. An intense red color develops (maximum, 495 m$\mu$).

## XI. Fumigants

Many of the halogen-containing fumigants may be determined by standard methods of halogen analysis and most of these methods will not be mentioned (see section on organic halogen methods).

### *1. Acrylonitrile*

Peterson and Radke (359) have described a method for the determination of this compound in air. Acrylonitrile is absorbed in concentrated sulfuric acid, neutralized with sodium hydroxide solution, hydrogen peroxide is added, and the solution is refluxed to form ammonia. The ammonia is absorbed in standard acid and determined by back-titration. A polarographic method has also been suggested for acrylonitrile (39).

### *2. Inorganic Cyanides [HCN and Ca(CN)$_2$]*

In Sinclair and Ramsey's method (405), the cyanide is acidified, distilled as hydrocyanic acid, and absorbed in alkali. Sodium isopurpurate is formed by the reaction of the sodium cyanide with sodium picrate and the absorbance is measured at 500 m$\mu$. Epstein (138) described a procedure in which cyanide ion is oxidized to cyanogen chloride with chloramine-T. The cyanogen chloride, on treatment with a specially prepared pyridine-pyrazalone reagent, yields a blue dye whose absorbance is measured at 620 m$\mu$. Kruse and Mellon (277) have adapted this method to determine both cyanide and thiocyanate.

### *3. Carbon Disulfide*

Carbon disulfide may be determined by the methods of Morehead (335) and Viles (439). In their procedures, carbon disulfide vapor is absorbed in a solution containing diethylamine and cupric acetate to give the yellow copper diethyl dithiocarbamate whose absorbance is measured.

### *4. Chloropicrin*

Feinsilver and Oberst (148) determined microquantities of chloropicrin by decomposition of the compound in a hot alkaline peroxide medium to yield nitrite ion. The nitrite ion was reacted with sulfanilic acid, the product was coupled with *N*-(1-naphthyl)ethylenediamine and the color was measured at 540 m$\mu$. Residues in grain may be determined by passing air through treated grain to elute the chloropicrin followed by absorption of the vapors in an absorbing solution (197).

### *5. Dichloronitroethane*

Jones and Riddick (260) determined this material by decomposition of the nitroparaffin to nitrous acid with concentrated sulfuric acid. The nitrous acid was reacted with resorcinol to form what is believed to be a nitrosophenolic compound and the color was measured at 560 m$\mu$. Most secondary and a few tertiary nitroparaffins interfere. As little as 3 to 5 $\gamma$ may be determined with an accuracy of ±5%. The method was adapted to determine halogenated nitroparaffin fumigants in grains by aeration of the sample and passage of the air through sulfuric acid scrubbers (260).

### *6. Ethylene Chlorobromide*

Sinclair and Crandall (404) determined this compound by hydrolysis with monoethanolamine to yield bromide ion. This could be determined either by titration, or by oxidation with chromic acid to bromine which could be determined colorimetrically.

### 7. *Ethylene Dibromide*

This compound may be absorbed in ethanolamine and the bromide ion formed determined (403). Kennett (262) has described methods for both ethylene chlorobromide and dibromide which depend on formation of halide ion.

### 8. *Ethylene Oxide*

El Khishen (133) described methods of recovering ethylene oxide from fumigated substances by passing air through them and then through a solution of acidified magnesium chloride. The excess acid was determined by back-titration. In the method of Gunther *et al.* (199) for Aramite, a sensitive method for ethylene oxide is described.

### 9. *Methyl Bromide*

Methyl bromide may be determined by methods described for halogen but Phillips and Bulger (361) have developed a rapid and accurate method for determination of methyl bromide in air by measurements of thermal conductivity.

### 10. *Trichloroacetonitrile*

This compound may be determined (299) by distillation of ammonia from an alkaline solution. The ammonia is absorbed in acid and the excess acid is back-titrated.

## XII. Inorganic Pesticides

The residue methods for inorganic insecticides and fungicides are well known, and many of them have been thoroughly evaluated through cooperative studies. Procedures have been given in detail in the Official Methods of Analysis of Agricultural Chemists (17). Where these methods are available they are briefly mentioned in this section under the individual elements. Usually an ashing, wet oxidation or other oxidation procedure is employed to obtain the element in a suitable form for the analysis.

### A. ARSENIC COMPOUNDS

The arsenic is eventually reduced to arsine in the presence of acid and zinc. The gas is passed over a paper strip impregnated with mercuric bromide to form a colored compound and this is estimated by comparison with standard stains (17, pp. 396–400). An alternate method is to convert the arsenic to arsenious chloride, which can be determined by a bromate titration (17, pp. 396–400). These methods may be applied to materials such as calcium arsenate, lead arsenate, Paris green, and to organic arsenic-containing compounds such as arsenomethane disulfide.

### B. COPPER COMPOUNDS

The copper is finally converted to the dithizone complex in carbon tetrachloride. The copper ion in turn is removed from the dithizone-carbon tetrachloride with mercuric chloride solution and determined as its diethyl dithiocarbamate by measurement at 440 m$\mu$. This method (17, pp. 402–405) may be applied to materials such as Bordeaux mixture, copper carbonate, copper-zinc chromate, copper oxide, and to organic copper compounds such as copper 8-hydroxyquinolinate, and copper naphthenates.

### C. FLUORINE COMPOUNDS

The general method (17, pp. 415–424) specifies ashing the sample with calcium hydroxide as a fluoride fixative, isolation of fluoride, and determination of the latter by the thorium-alizarin lake procedure. For apples and pears, stripping with dilute hydrochloric acid simplifies the method. The method may be applied to pesticides such as cryolite, sodium fluoride, zinc fluoroarsenate and barium silicofluoride.

### D. LEAD COMPOUNDS

The general method (17, pp. 424–438) calls for ashing the sample to destroy organic matter and solution of the residue in hydrochloric acid. After the addition of ammonium citrate and potassium cyanide, and adjustment of the pH to 9.5–10, the lead is extracted into dithizone-chloroform. Absorbance measurements are made at 510 m$\mu$. The method may be used to determine lead arsenate.

### E. MERCURY COMPOUNDS

Mercury is determined as the dithizone complex (17, pp. 438–442) (389). Klein (268) determined micro amounts of mercury in food and found it imperative to digest the sample completely in a specially-designed apparatus. Klein (269) also described a procedure for elimination of interferences due to copper. Polley and Miller (367) described a rapid micro-procedure for mercurial fungicides in soils. A sulfuric acid-hydrogen peroxide oxidizing mixture is substituted for the usual nitric acid-sulfuric acid mixture. These methods may be applied to organic mercury fungicides.

### F. SELENIUM COMPOUNDS

The procedure consists of wet digestion with nitric and sulfuric acids in the presence of a mercuric oxide fixative. Selenium is distilled as its volatile bromide which is then reduced to selenium with sulfur dioxide. The elemental selenium is reoxidized to selenious acid and determined by titration with standard thiosulfate and standard iodine (17, pp. 447–451). The method may be used for sodium selenate.

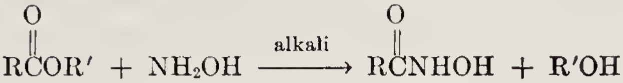
### G. SULFUR COMPOUNDS

Gunther *et al.* (194) described a procedure in which the sulfur-containing residue is oxidized to sulfate by alkaline fusion and determined gravimetrically. Gunther and Blinn (197, pp. 596–598) have modified a colorimetric method described by Bartlett and Skoog (31) in which an aliquot of a petroleum ether solution of the sulfur is mixed with acetone and treated with cyanide to yield thiocyanate. The latter is determined by measurement at 465 m$\mu$ after addition of an acetone solution of ferric chloride.

## XIII. Pesticides of Natural Origin and Their Synergists

### A. PYRETHROIDS

Lord (293) described a color test applicable to the pyrethrins based on their reaction with hydroxylamine to form hydroxamic acids which yield colored complexes with ferric ions. This reaction was applied by Edwards and Cueto (129) to the estimation of pyrethrins on coated paper bags. A pyrethrin standard, preferably the same as used in the original application is employed. The range studied was 1 to 3 mg. The reaction is a nonspecific, general one given by most esters and therefore can only be applied to pyrethroids in the absence of other esters:



$$R\overset{\overset{O}{\|}}{C}OR' + NH_2OH \xrightarrow{\text{alkali}} R\overset{\overset{O}{\|}}{C}NHOH + R'OH$$

Cueto and Dale (100), with the Feinstein test (149) as a lead, developed a colorimetric method for the determination of pyrethrins, allethrin and furethrin based on reaction with sodium sulfide in aqueous ethanol and measurement at 540 m$\mu$. The average error was about ±4% and the method was applied to treated paper with good results.

Levy and Estrada (287) modified the Feinstein test and developed a colorimetric method for the pyrethrins based on reaction with sulfur in alcoholic potassium hydroxide-carbon tetrachloride solution. A 560 m$\mu$ filter is used for amounts of pyrethrins between 0.2 and 1.0 mg. per ml., and a 440 m$\mu$ filter for amounts between 0.04 and 0.2 mg. DDT and the commonly used synergists do not interfere.

Schreiber and McClellan (397) utilized the color produced by a modified Denigès reagent (mercuric oxide dissolved in sulfuric acid) to determine pyrethrins, allethrin and other pyrethroids. Since the test depends on reaction with the chrysanthemum monocarboxylic acid portion of the molecule, only pyrethrin I and cinerin I in the pyrethrins are determined. The method is sensitive to about 5 $\gamma$ and pyrethrins or allethrin may be determined in treated paper, cloth, or flour. Much of the difficulty in the determination of pyrethroids in impregnated paper lies in the difficulty of extraction due possibly to strong adsorption or decomposition of the insecticide.

Williams *et al.* (449) described a simple, rapid colorimetric method for pyrethrins based on heating the sample in 20% ethyl acetate—80% orthophosphoric acid for 3 minutes in a boiling-water bath and measurement of the color at 550 m$\mu$. Grains, burlap, and cardboard contain substances that reduce the color and internal standards are recommended to overcome this. It is interesting that allethrin, the cinerins and most other insecticides do not give the test, whereas pyrethrins I and II do. Therefore the pentadiene side chain must be involved in production of the color.

Green and Schechter (187) described an analytical assay method, based on the formation of the 2,4-dinitrophenylhydrazone of allethrin and chromatography of the derivative, which probably could be adapted to residue determinations. Moore (334) also utilized the dinitrophenylhydrazone derivatives of pyrethrins and allethrin in his method but in this case the molecule is split in contrast to Green and Schechter's method in which the derivative of the intact allethrin molecule is prepared.

Schechter *et al.* (393) isolated the crystalline *alpha dl-trans* isomer of allethrin which they suggested could be used as a standard in chemical methods and this has been done to some extent (100,397).

Winteringham (452) developed a reversed phase chromatographic procedure for the separation and detection of pyrethroids and their derivatives and Bergamini and Versorese (36) have utilized circular chromatography.

Polarographic (349,350,459) and spectrophotometric (158,402) studies have been made on the pyrethrins and allethrin but it is doubtful that these methods can be used for residues because of the presence of interferences.

The work of Freeman (159,160) who investigated the stability of allethrin and pyrethrins to storage and heat will be of interest to those interested in possible modes of decomposition of these substances, as will the work of Blackith (41).

## B. PYRETHROID SYNERGISTS

### 1. *Piperonyl Butoxide*

Jones *et al.* (257) modified the Labat test (280) and developed a colorimetric method for piperonyl butoxide. The latter produces a blue color when heated with tannic acid in an acetic acid-phosphoric acid mixture. The tannic acid must be specially purified. The test is reasonably specific for piperonyl butoxide since many other compounds or synergists containing the methylenedioxyphenyl group, and by-products in technical piperonyl butoxide, do not give the test. Pyrethrins at greater than a 1:10 ratio cause a reduction in color. This may be overcome (388) by preliminary saponification or by chromatography. Partition between 85–90% aqueous methanol and hexane may also be used for isolation of the butoxide. The method can be applied to paper-coatings, grains, and feces and urine from experimental animals. Williams and Sweeney (450) developed a chromatographic separation on Florisil as an adsorbent which will extend the usefulness of the Jones procedure down to 2 p.p.m. on vegetables and grains with better than 95% recoveries.

Blum (49) also modified the Labat test (280) for methylenedioxyphenyl compounds so that it can be applied to the commonly-used pyrethrum synergists containing this group. The synergist (5–80 $\gamma$), dissolved in acetonitrile is mixed with gallic acid and sulfuric acid and the green color which develops is measured colorimetrically at 660 m$\mu$. The standard deviation is about ±4%. The mechanism of the reaction depends on liberation of formaldehyde (from methylenedioxy groups) which reacts with the gallic acid in the presence of sulfuric acid. All methylenedioxyphenyl-containing compounds give the test.

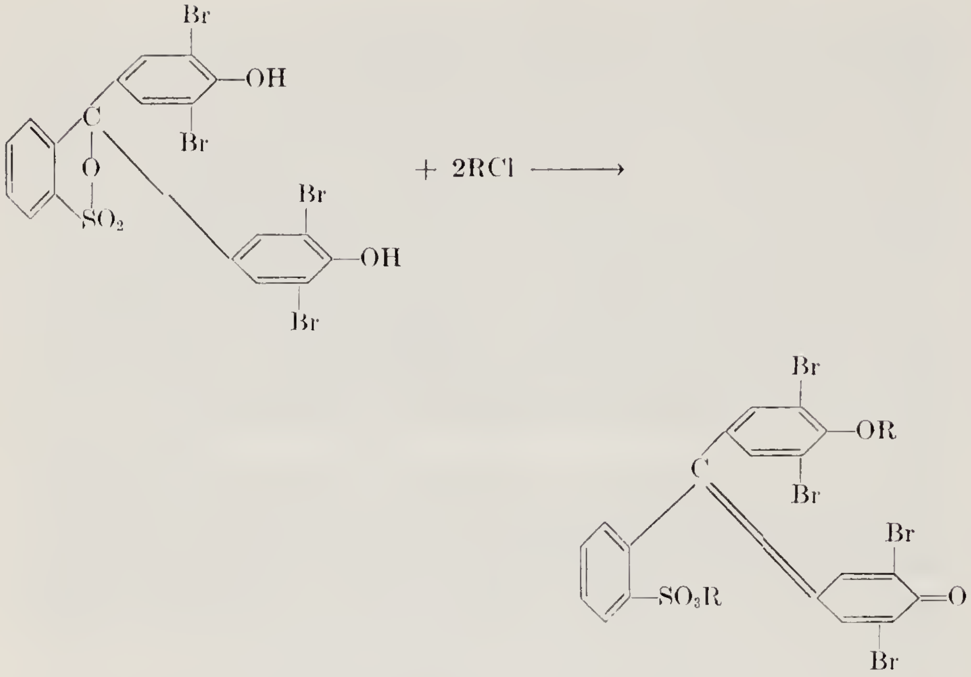
Beroza (37) developed a method for methylenedioxyphenyl-containing synergists in fly sprays and aerosols based on liberation of formaldehyde and production of a color with chromotropic acid in sulfuric acid. This method possibly could be adapted for residues in conjunction with an adequate cleanup procedure.

### C. ROTENONE AND OTHER ROTENOIDS

Jones (256) has reviewed analytical methods up to 1942. Subba Rao and Pollard (424) claimed that, of a number of colorimetric tests, the Goodhue modification (183) of the Gross-Smith method (189) for rotenone and rotenoids was the only reliable quantitative method. The test depends on treatment of an acetone solution of the rotenoid with alcoholic potassium hydroxide-sodium nitrite solution and then with dilute sulfuric acid to give a red color. They studied the method in some detail and applied it to spray residues, which contained rotenone, and the decomposition products dehydrorotenone and rotenonone.

### D. ALKALOIDS AND ALKALOID-CONTAINING PESTICIDES

A number of the methods for alkaloids depend on a dye-complex technique. Auerbach (19) studied this technique for quaternary ammonium compounds which is based on the formation of complex salts with colored indicators or dyes. The dye salts of quaternary ammonium compounds can be readily extracted from alkaline solutions into organic solvents, particularly the chlorinated ones. Auerbach employed bromophenol blue with ethylene dichloride as the solvent and the reaction is given in equation (XXIII). It was found



R = Quaternary ammonium cation

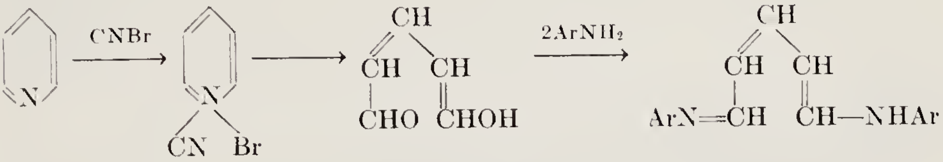
(XXIII)

that most simple primary, secondary, and tertiary amines and alkaloids gave a negative test. In an alkaline medium, the dye salts of alkaloids are not extractable; however, these salts become extractable from acid solutions and minute amounts of a number of alkaloids can thus be determined.

This procedure was used by Prudhomme (371) for quinine. Methods have also been developed for strychnine by Auerbach (19), for nicotine, strychnine and other bases in tissues (175), and for many other naturally occurring or synthetic organic bases. The dye-complex technique will be referred to again under nicotine and other individual pesticides. Some precautions to be observed with these dye-complex procedures have been described (17, pp. 496–503) (285). The same technique can be employed for the determination of certain high molecular weight acidic compounds if basic dyes are used.

### *1. Nicotine by Colorimetric Methods*

A sensitive colorimetric procedure for nicotine and related bases having a pyridine ring has been used extensively. It is based on reaction of nicotine with cyanogen bromide followed by coupling with an aromatic amine, usually beta-naphthylamine. The nicotine is steam-distilled and amounts above 2γ per ml. of distillate can be determined with a ±1% error (431). The reaction is given in equation (XXIV). Wolff *et al.* (454) determined 1 to 50γ of nicotine in blood by this procedure. Markwood (302) stripped apples with dilute sodium hydroxide solution, purified the extract by means of calcium bentonite coagulate, and measured the color produced with cyanogen bromide and beta-naphthylamine at 490 mμ. The optimum conditions for the color reactions have been studied by Markwood (303). Fabre *et al.* (142) have applied the cyanogen bromide method, with benzidine as the aromatic amine, to the determination of nicotine in plant residues.



(XXIV)

The cyanogen bromide method does not distinguish nicotine from nornicotine and certain other organic bases; however, by utilizing the colors produced by cyanogen bromide alone without the use of an aromatic amine, Larson and Haag (282) developed a colorimetric procedure for the simultaneous determination of nicotine and nornicotine based on the yellowish-green color (375 mμ maximum) given by nicotine and the pink color (540 mμ maximum) given by nornicotine with the reagent.

Gettler and Sunshine (175) determined microgram quantities of nicotine and other alkaloids in human tissues by means of a methyl orange dye-complex method and Rinthakul and Hannen (379) used a bromothymol blue method.

A method based on the color produced by reduction of nicotine silicomolybdate has been applied to soil analysis (427).

Ramamoorthy *et al.* (374) determined nicotine by means of the ultraviolet maximum at 259 mμ in acid solution, and Willits *et al.* (453) also utilized this method and corrected for background absorption, but these procedures have not been applied to residues. Jeffrey and Eoff (251) and Tso and Jeffrey (432) have developed

## TABLE I

Range of Tolerances[a, b]

| Pesticide | P.p.m. |
|---|---|
| Aldrin | 0–0.75 |
| Allethrin | 4 (post-harvest use) exempt on growing crops |
| Aramite | 1 |
| Benzene hexachloride (Tech.) | 5 |
| Bromide, inorganic from soil treatment and fumigation with ethylene dibromide and fumigation with methyl bromide | 5–200 |
| Calcium arsenate | 3.5 ($As_2O_3$) |
| Calcium cyanide | 0–25 (HCN) |
| Captan | 20 |
| Chlordane | 0.3 |
| Chlorobenzilate | 5 |
| 3-(*p*-Chlorophenyl)-1,1-dimethylurea | 1 |
| Chlortetracycline | 7 |
| Copper arsenate | 3.5 ($As_2O_3$) |
| DDT | 7 |
| 1,1-Dichloro-2,2-bis(*p*-ethylphenyl) ethane | 15 |
| 2,4-Dichlorophenoxyacetic acid | 5 |
| 2,4-Dichlorophenoxyethyl sulfate, sodium | 2–6 |
| 3-(3,4-Dichlorophenyl)-1,1-dimethylurea | 1–2 |
| Dichlone (Phygon) | 3 |
| Dicyclohexylamine salt of dinitro-*o*-cyclohexylphenol | 1 |
| Dieldrin | 0–0.75 |
| Dinitro-*o*-cyclohexylphenol | 1 |
| Dinitro-*o*-*sec.*-butylphenol | 0 |
| Dinitro-*o*-cresol | 0 |
| Diphenyl | 110 |
| Endrin | 0 |
| EPN | 0.5–3.0 |
| Ethylene oxide | 50 |
| Ferbam | 0.1–7.0[c] |
| Fluorine-containing compounds | 7 (combined fluorine) |
| Glyodin | 5 |
| Heptachlor | 0.1 |
| Hexaethyl tetraphosphate | 0 |
| Hydrogen cyanide | 0–250 |
| Lead arsenate | 1–7 (lead) |
| Lindane | 10 |
| Magnesium arsenate | 3.5 ($As_2O_3$) |
| Malathion | 8 |

*(continued)*

TABLE I (*continued*)

| Pesticide | P.p.m. |
|---|---|
| Maneb | 0.1–7[c] |
| Mercury-containing compounds | 0 (mercury) |
| Metacide (methyl parathion) | 1 |
| Methoxychlor | 2–100 |
| Mineral oil | 200 (post-harvest use) exempt on growing crops |
| Naphthalene acetic acid | 1 |
| Nicotine-containing compounds | 2 (nicotine) |
| Ovex | 3–5 |
| Oxytetracycline | 7 |
| Parathion | 1 |
| Phenothiazine | 7 |
| Piperonyl butoxide | 8–20[d] |
| Pyrethrins | 1–3[d] |
| Selenium-containing compounds | 0 (Se) |
| Sodium arsenate | 3.5 ($As_2O_3$) |
| Sodium 2,2-dichloropropionate (as dichloropropionic acid) | 5–35 |
| Sodium-*o*-phenylphenate | 10 (*o*-phenylphenol) |
| Sulphenone | 8 |
| Systox | 0.3–1.25 |
| Tartar emetic | 3.5 ($Sb_2O_3$) |
| TDE | 7 |
| Tetraethyl pyrophosphate | 0 |
| Thiram | 3 |
| Toxaphene | 7 |
| Zineb | 1–60 |
| Ziram | 0.1–7[c] |

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[a] Tolerances established for related pesticide chemicals may limit the amount of a common component (such as $As_2O_3$) that may be present, or may limit the amount of biological activity (such as cholinesterase inhibition) that may be present, or may limit the total amount of related pesticide chemicals (such as chlorinated hydrocarbons) that may be present; see the regulations for details (Federal Register of March 11, 1955, and of February 4 and 21, 1956).

[b] The data on residue tolerances were compiled by L. L. Ramsey, Department of Health, Education and Welfare, Food and Drug Administration, Washington, D. C.

[c] Calculated as Zineb.

[d] Applies to various stored grains; on growing crops the material is exempt from tolerance.

paper chromatographic methods for the determination of alkaloids in tobacco, but this too has not been applied to residues.

Nornicotine interferes with most methods for nicotine but may be determined by the method of Larson and Haag (282) or by the method of Feinstein and McCabe (150). Nicotine and anabasine may be separated chromatographically (241).

### 2. *Nicotine by Precipitation Methods*

Nicotine forms an insoluble precipitate with silicotungstic acid and solutions containing 0.1–0.5 mg. of nicotine can be analyzed with an average accuracy of $\pm 2\gamma$ (412). Nephelometric determination of the silicotungstate (411) or the phosphotungstate (304) is also possible. The silicotungstate method is subject to serious errors when large amounts of ammonia or ammonium salts are present (348). Robinson (382) determined nicotine in the presence of nornicotine turbidimetrically as the iodomercurate.

Ralston (373) determined nicotine on apples and apple foliage by stripping with a mixture of dilute sodium hydroxide solution and ethylene dichloride from which the nicotine is recovered and determined by distillation and silicotungstic acid precipitation. The results are accurate to 0.1 mg. on amounts up to 12 mg. of nicotine in a 1-kg. sample.

### 3. *Ryanodine*

A test has been found for ryanodine (from Ryania extract), which gives a purple color when heated with 1% alcoholic *p*-dimethylaminobenzaldehyde and dilute sulfuric acid but this has not been developed into a residue method (390).

### 4. *Strychnine*

Strychnine may be determined as described by Snell and Snell (410), or by Auerbach's method (19,175).

## E. ANTIBIOTICS

Antibiotics are coming into wider use against fireblight of apples and pears, cherry leaf spot, and other bacterial and fungous infections of plants. The methods for residues are mainly microbiological assays such as for cycloheximide (Actidione) on cherries (370). However, streptomycin may be determined colorimetrically (147,

314) by alkaline hydrolysis to maltol which gives a red color (540 mμ maximum) with ferric ion.

## XIV. Pesticide Tolerances

Table I gives a summary of the maximum tolerances or range of tolerances for various pesticidal residues as established under the Miller law. The tolerance for any pesticide may vary with the crop or commodity. The following pesticide chemicals are exempt from the requirement of a tolerance when applied to growing crops before harvest: Bordeaux mixture, copper acetate, basic copper carbonate (malachite), copper-lime mixtures, copper oxychloride, copper silicate, copper sulfate, basic copper sulfate, copper-zinc chromate, cuprous oxide, MGK 264 (*N*-(2-ethylhexyl)bicyclo[2.2.1]-5-heptene-2,3-dicarboximide), petroleum oils, piperonyl butoxide, piperonyl cyclonene, propyl isomer, pyrethrum and pyrethrins, rotenone or derris or cube roots, ryania, and sabadilla. The following pesticides are exempted from the requirement of a tolerance when used as fumigants on certain grains: carbon bisulfide, carbon tetrachloride, ethylene dichloride, ethylene dibromide (resulting inorganic bromide residues are covered by tolerances), and ammonia when used as a preservative on citrus.

## References

1. Agazzi, E. J., Peters, E. D., and Brooks, F. R., *Anal. Chem.*, *25*, 237 (1953).
2. Alder, K., and Stein, G., *Ann.*, *485*, 211 (1931).
3. Aldridge, W. N., *Biochem. J.*, *53*, 117 (1953).
4. Aldridge, W. N., *Chemistry & Industry*, *1954*, 473.
5. Aldridge, W. N., and Barnes, J. M., *Nature*, *169*, 345 (1952).
6. Allessandrini, M. E., *Ann. chim. appl.*, *38*, 414 (1948).
7. Allen, R. J. L., *Biochem. J.*, *34*, 858 (1940).
8. Alles, G. A., and Hawes, R. C., *J. Biol. Chem.*, *133*, 375 (1940).
9. Alrose Chemical Co., Division of Geigy Chemical Corp., *Methods of Analysis for Diazinon Residues*, unpublished (1956).
10. American Cyanamid Co., *Determination of Residual Insecticide 4124 in Milk.*
11. American Cyanamid Co., *Thimet Insecticide Technical Manual*, April 1956.
12. American Society for Testing Materials, Standards on Petroleum Products and Lubricants, Designation D-2, p. 21 (1951).
13. Ammon, R., *Pflügers Arch. ges. Physiol.*, *233*, 486 (1933).
14. Amsden, R. C., and Walbridge, D. J., *J. Agr. Food Chem.*, *2*, 1323 (1954).
15. Ard, J. S., *Anal. Chem.*, *20*, 858 (1948).
16. Armstrong, G., Bradbury, F. R., and Standen, H., *Ann. Appl. Biol.*, *38*, 555 (1951).

17. Association of Official Agricultural Chemists, *Official Methods of Analysis*, 8th ed., 1955.
18. Auerbach, M. E., *Anal. Chem., 22,* 1287 (1950).
19. Auerbach, M. E., *Ind. Eng. Chem., Anal. Ed., 15,* 492 (1943); *16,* 739 (1944).
20. Augustinsson, K. B., *Acta Physiol. Scand., 15, Suppl. 52* (1948).
21. Avens, A. W., Chapman, P. J., and Pearce, G. W., *J. Econ. Entomol., 41,* 432 (1948).
22. Averell, P. R., and Norris, M. V., *Anal. Chem., 20,* 753 (1948).
23. Babers, F. H., and Pratt, J. J., *Physiol. Zoöl., 23,* No. 1, 58 (1950).
24. Baernstein, H. D., *Ind. Eng. Chem., Anal. Ed., 15,* 251 (1943).
25. Bailes, E. L., and Payne, M. G., *Ind. Eng. Chem., Anal. Ed., 17,* 438 (1945).
26. Bandurski, R. S., *Botan. Gaz., 108,* 446 (1947).
27. Bann, J. M., DeCino, T. J., Earle, N. W., and Sun, Y. P., *J. Agr. Food Chem., 4,* 937 (1956).
28. Barlow, F., *Nature, 160,* 719 (1947).
29. Barnes, J. M., *Chemistry & Industry, 1954,* 478.
30. Barrons, K. C., and Hummer, R. W., *Agr. Chemicals, 6,* No. 6, 48 (1951).
31. Bartlett, J. K., and Skoog, D. A., *Anal. Chem., 26,* 1008 (1954).
32. Beard, R. L., *J. Econ. Entomol., 44,* 469 (1951).
33. Bennet-Clark, T. A., Tambiah, M. S., and Kefford, N. P., *Nature, 169,* 452 (1952).
34. Berck, B., *Anal. Chem., 25,* 1253 (1953).
35. Berenblum, J., and Chain, E., *Biochem. J., 32,* 295 (1938).
36. Bergamini, C., and Versorese, W., *Sperimentale, Sez. chim. biol., 5,* 6 (1954).
37. Beroza, M., *J. Agr. Food Chem., 4,* 53 (1956).
38. Biggs, A. I., *Analyst, 80,* 279 (1955).
39. Bird, W. L., and Hale, C. H., *Anal. Chem., 24,* 586 (1952).
40. Bissinger, W. E., and Fredenburg, R. H., *J. Assoc. Offic. Agr. Chemists, 34,* 812 (1951).
41. Blackith, R. E., *J. Sci. Food Agr., 3,* 219, 224, 482 (1952).
42. Blaedel, W. J., Lewis, W. B., and Thomas, J. W., *Anal. Chem., 24,* 509 (1952).
43. Bleidner, W. E., *J. Agr. Food Chem., 2,* 682 (1954).
44. Bleidner, W. E., Baker, H. M., Levitsky, M., and Lowen, W. K., *J. Agr. Food Chem., 2,* 476 (1954).
45. Blinn, R. C., and Gunther, F. A., *Anal. Chem., 22,* 1219 (1950).
46. Blinn, R. C., and Gunther, F. A., *J. Agr. Food Chem., 3,* 1013 (1955).
47. Blinn, R. C., Gunther, F. A., and Kolbezen, M. J., *J. Am. Chem. Soc., 74,* 5516 (1952).
48. Blinn, R. C., Gunther, F. A., and Kolbezen, M. J., *J. Agr. Food Chem., 2,* 1080 (1954).
49. Blum, M. S., *J. Agr. Food Chem., 3,* 122 (1955).
50. Bost, R. W., and Nicholson, F., *Ind. Eng. Chem., Anal. Ed., 7,* 190 (1935).
51. Bradbury, F. R., Higgons, D. J., and Stoneman, J. P., *J. Soc. Chem. Ind. (London), 66,* 65 (1947).
52. Bradbury, F. R., and Standen, H., *J. Sci. Food Agr., 5,* 252 (1954).
53. Brauer, R. W., *J. Pharmacol. Exptl. Therap., 92,* 162 (1948).
54. Brokke, M. E., Kiigemagi, U., and Terriere, L. C., Abstracts of Papers,

129th Meeting of the American Chemical Society, Dallas, Texas, April 1956.
55. Brown, H. V., and Bush, A. F., *Arch. Ind. Hyg. and Occupational Med.*, *1*, 633 (1950).
56. Buckley, R., and Colthurst, J. P., *Analyst*, *79*, 285 (1954).
57. Burchfield, H. P., and McNew, G. L., *Phytopathology*, *38*, 299 (1948).
58. Burchfield, H. P., and McNew, G. L., *Phytopathology*, *38*, 665 (1948).
59. Burchfield, H. P., and Storrs, E. E., *Contribs. Boyce Thompson Inst.*, *17*, 333 (1953).
60. Callan, T., and Strafford, N., *J. Soc. Chem. Ind. (London)*, *43*, 8T (1924).
61. Canbäck, T., and Zajaczkowska, H., *J. Pharm. and Pharmacol.*, *2*, 545 (1950).
62. Carbide and Carbon Chemicals Co., Glyodin, Chemical Method 30-J1C2-1, Nov. 1954.
63. Carbide and Carbon Chemicals Co., Natrin, Chemical Methods 30-B32S2-6a (1955).
64. Carbide and Carbon Chemicals Co., Crag Herbicide 1, Chemical Methods, 30-B32S1-7 (1954); 30-B32S1-8a (1955).
65. Carbide and Carbon Chemicals Co., Dichloralurea, Chemical Methods, 30-21Cl-3; 3021Cl-4b; 30-21Cl-5a (1955).
66. Carman, G. E., Gunther, F. A., Blinn, R. C., and Garmus, R. D., *J. Econ. Entomol.*, *45*, 767 (1952).
67. Carter, R. H., and Hubanks, P. E., unpublished report.
68. Carter, R. H., *Anal. Chem.*, *19*, 54 (1947).
69. Carter, R. H., and Hubanks, P. E., *J. Assoc. Offic. Agr. Chemists*, *29*, 112 (1946).
70. Carter, R. H., Nelson, R. H., and Gersdorff, W. A., *Advances in Chem. Ser.*, *No. 1*, 271 (1950).
71. Casida, J. E., *Biochem. J.*, *60*, 487 (1955).
72. Casida, J. E., *J. Physiol. (London)*, *127*, 20P (1954).
73. Casida, J. E., *Science*, *122*, 597 (1955).
74. Casida, J. E., Allen, T. C., and Stahmann, M. A., *J. Am. Chem. Soc.*, *74*, 5548 (1952).
75. Casida, J. E., Allen, T. C., and Stahmann, M. A., *J. Biol. Chem.*, *210*, 607 (1954).
76. Casida, J. E., Allen, T. C., and Stahmann, M. A., *Nature*, *172*, 243 (1953).
77. Casida, J. E., Chapman, R. K., and Allen, T. C., *J. Econ. Entomol.*, *45*, 568 (1952).
78. Casida, J. E., Chapman, R. K., Stahmann, M. A., and Allen, T. C., *J. Econ. Entomol.*, *47*, 64 (1954).
79. Casida, J. E., Gatterdam, P. E., Getzin, L. W., and Chapman, R. K., *J. Agr. Food Chem.*, *4*, 236 (1956).
80. Casida, J. E., and Stahmann, M. A., *J. Agr. Food Chem.*, *1*, 883 (1953).
81. Chanin, M., *Science*, *119*, 323 (1954).
82. Chemagro Corp., New York, N. Y., unpublished.
83. Chemagro Corp., New York, N. Y., *Colorimetric Determination of Chlorthion Residues on Fruit and Vegetables.*

84. Cholak, J., and Hubbard, D. M., *Ind. Eng. Chem., Anal. Ed., 18,* 149 (1946).
85. Claborn, H. V., *J. Assoc. Offic. Agr. Chemists, 29,* 330 (1946).
86. Claborn, H. V., and Beekman, H. F., *Anal. Chem., 24,* 220 (1952).
87. Claborn, H. V., and Patterson, W. I., *J. Assoc. Offic. Agr. Chemists, 29,* 206 (1946).
88. Clarke, D. G., Baum, H., Stanley, E. L., and Hester, W. F., *Anal. Chem., 23,* 1842 (1951).
89. Clifford, P. A., *J. Assoc. Offic. Agr. Chemists, 29,* 195 (1946).
90. Clifford, P. A., *J. Assoc. Offic. Agr. Chemists, 30,* 337 (1947).
91. Clifford, P. A., *J. Assoc. Offic. Agr. Chemists, 38,* 673 (1955).
92. Conway, E. J., *Microdiffusion Analysis and Volumetric Error,* 3rd ed., Chap. XXII, XXIII, XXIV, Crosby Lockwood, London, 1950.
93. Cook, J. W., *J. Assoc. Offic. Agr. Chemists, 36,* 569 (1953).
94. Cook, J. W., *J. Assoc. Offic. Agr. Chemists, 37,* 561 (1954).
95. Cook, J. W., *J. Assoc. Offic. Agr. Chemists, 37,* 984, 987, 989(1954).
96. Cook, J. W., *J. Assoc. Offic. Agr. Chemists, 38,* 150 (1955).
97. Cook, J. W., *J. Assoc. Offi. Agr. Chemists, 38,* 826 (1955).
98. Cranham, J. E., Higgons, D. J., and Stevenson, H. A., *Chemistry & Industry, 1953,* 1206.
99. Cristol, S. J., *J. Am. Chem. Soc., 69,* 333 (1947).
100. Cueto, C., and Dale, W. E., *Anal. Chem., 25,* 1367 (1953).
101. Dahm, P. A., *Chem. Specialties Mfrs. Assoc. Proc.,* p. 71 (May 1953).
102. Dahm, P. A., Fountaine, F. C., Pankaskie, J. E., Smith, R. C., and Atkeson, F. W., *J. Dairy Sci., 33,* 747 (1950).
103. Dahm, P. A., and Jacobson, N. L., *J. Agr. Food Chem., 4,* 150 (1956).
104. Danish, A. A., and Lidov, R. E., *Anal. Chem., 22,* 702 (1950).
105. Danish, A. A., and Lidov, R. E., *Advances in Chem.* Ser., *No. 1,* 190 (1950).
106. David, A., Hartley, G. S., Heath, D. F., and Pound, D. W., *J. Sci. Food Agr., 2,* 310 (1951).
107. Davidow, B., *J. Assoc. Offic. Agr. Chemists, 33,* 130 (1950).
108. Davidow, B., *J. Assoc. Offic. Agr. Chemists, 33,* 886 (1950).
109. Davidow, B., and Frawley, J. P., *Proc. Soc. Exptl. Biol. Med., 76,* 780 (1951).
110. Davidow, B., and Radomski, J. L., *J. Pharmacol. Exptl. Therap., 107,* 259 (1953).
111. Davidow, B., and Woodard, G., *J. Assoc. Offic. Agr. Chemists, 32,* 751 (1949).
112. Davies, D. R., and Nicholls, J. D., *Brit. Med. J.,* 1373 (1955).
113. Davis, F. L., and Selman, F. L., *Weeds, 3,* 11 (1954).
114. Derkosch, J., and Mayer, F. X., *Mikrochim. Acta,* p. 495 (1955); through *Chem. Abstracts, 49,* 11508 (1955).
115. Dickinson, D., *Analyst, 71,* 327 (1946).
116. Diggle, W. M., and Gage, J. C., *Biochem. J., 49,* 491 (1951).
117. Diggle, W. M., and Gage, J. C., *Nature, 168,* 998 (1951).
118. DiStefano, V., and Hurwitz, L., *Arch. Biochem. and Biophys., 47,* 228 (1953).
119. Dow Chemical Co., Determination of Small Amounts of TCA, Analytical Method MLE. 53.5 (1953).
120. Dow Chemical Company, Modification of the Determination of Bis(*p*-

chlorophenoxy)methane (K-1875) in Spray Residues on Fresh Fruit (Aug. 1951).

121. Dowden, H. C., *Biochem. J.*, *32*, 455 (1938).
122. Downing, G., and Norton, L. B., *Anal. Chem.*, *23*, 1870 (1951).
123. DuBois, K. P., and Cotter, G. J., *Am. Med. Assoc. Arch. Ind. Health*, *11*, No. 1, 53 (1955).
124. DuBois, K. P., Doull, J., and Coon, J. M., *J. Pharmacol. Exptl. Therap.*, *99*, 376 (1950).
125. DuBois, K. P., and Mangun, G. H., *Proc. Soc. Exptl. Biol. Med.*, *64*, 137 (1947).
126. Dupée, L. F., Heath, D. F., and Otter, I. K. H., *J. Agr. Food Chem.*, *4*, 233 (1956).
127. Edwards, F. I., *Anal. Chem.*, *21*, 1415 (1949).
128. Edwards, F. I., *J. Assoc. Offic. Agr. Chemists*, *33*, 783 (1950).
129. Edwards, F. I., and Cueto, C., *Anal. Chem.*, *24*, 1357 (1952).
130. Edwards, F. I., and Hall, S. A., *Anal. Chem.*, *21*, 1567 (1949).
131. E. I. du Pont de Nemours & Co., Wilmington, Delaware, EPN, A Manual of Technical Data (July 1951).
132. E. I. du Pont de Nemours & Co., Wilmington, Delaware, Methoxychlor—A Summary of Analytical Methods (Feb. 1951).
133. El Khishen, S. A., *J. Sci. Food Agr.*, *1*, 71 (1950).
134. Ely, R. E., Moore, L. A., Carter, R. H., Hubanks, P. E., and Poos, F. W., *J. Dairy Sci.*, *37*, 1461 (1954).
135. Ely, R. E., Moore, L. A., Hubanks, P. E., Carter, R. H., and Poos, F. W., *J. Dairy Sci.*, *37*, 294 (1954).
136. Ely, R. E., Moore, L. A., Hubanks, P. E., Carter, R. H., and Poos, F. W., *J. Dairy Sci.*, *38*, 669 (1955).
137. Emerson, E., *J. Org. Chem.*, *8*, 417 (1943).
138. Epstein, J., *Anal. Chem.*, *19*, 272 (1947).
139. Erwin, W. R., Schiller, D., and Hoskins, W. M., *J. Agr. Food Chem.*, *3*, 676 (1955).
140. Ettinger, M. B., and Ruchhoft, C. C., *Anal. Chem.*, *20*, 1191 (1948).
141. Ewart, W. H., Gunther, F. A., Barkley, J. H., and Elmer, H. S., *J. Econ. Entomol.*, *45*, 578 (1952).
142. Fabre, R., Truhaut, R., and Boudene, C., III$^e$ *congr. intern. Phytopharmacie*, Paris, 1952; *Anal. Chem.*, *24*, 2014 (1952).
143. Fahey, J. E., Hamilton, D. W., and Rings, R. W., *J. Econ. Entomol.*, *45*, 700 (1952).
144. Fahey, J. E., and Rusk, H. W., *Anal. Chem.*, *23*, 1826 (1951).
145. Fairing, J. D., and Warrington, H. P., *Advances in Chem. Ser.*, *No. 1*, 260 (1950).
146. Fallscheer, H. O., and Cook, J. W., *J. Assoc. Offic. Agr. Chemists*, *39*, 691 (1956).
147. Federal Register, Section, 141b, 108(b).
148. Feinsilver, F., and Oberst, F. W., *Anal. Chem.*, *25*, 820 (1953).
149. Feinstein, L., *Science*, *115*, 245 (1952).
150. Feinstein, L., and McCabe, E. T., *Anal. Chem.*, *23*, 924 (1951).

151. Fenwick, M. L., and Parker, V. H., *Analyst, 80,* 774 (1955).
152. Fischer, W., *Z. anal. Chem., 131,* 192 (1950).
153. Fleisher, J. H., Jandorf, B. J., Summerson, W. H., and Norton, D. P., *Federation Proc., 9,* 171 (1950).
154. Fleisher, J. H., and Pope, E. J., *Arch. Ind. Hyg. and Occupational Med., 9,* 323 (1954).
155. Fleisher, J. H., Spear, S., and Pope, E. J., *Anal. Chem., 27,* 1080 (1955).
156. Frawley, J. P., Hagan, E. C., and Fitzhugh, O. G., *J. Pharmacol. Exptl. Therap., 105,* 156 (1952).
157. Freed, V. H., *Science, 107,* 98 (1948).
158. Freeman, S. K., *Anal. Chem., 27,* 1268 (1955).
159. Freeman, S. K., *Soap and Chem. Specialties, 32,* No. 2, 131 (1956).
160. Freeman, S. K., *Soap and Chem. Specialties, 32,* No. 3, 150 (1956).
161. Fujiwara, K., *Sitzber. Abhandl. naturfors. Ges. Rostock, 6,* 33 (1916); through *Chem. Abstracts, 11,* 3201 (1917).
162. Fukuto, T. R., and Metcalf, R. L., *J. Am. Chem. Soc., 76,* 5103 (1954).
163. Fukuto, T. R., Metcalf, R. L., March, R. B., and Maxon, M. G., *J. Econ. Entomol., 48,* 347 (1955).
164. Furman, N. H., and Low, G. N., *J. Am. Chem. Soc., 57,* 1585, 1588 (1935).
165. Gage, J. C., *Biochem. J., 54,* 426 (1953).
166. Gage, J. C., *Analyst, 75,* 189 (1950).
167. Gage, J. C., *Analyst, 77,* 123 (1952).
168. Gard, L. N., and Rudd, N. G., *J. Agr. Food Chem., 1,* 630 (1953).
169. Gard, L. N., Pray, B. O., and Rudd, N. G., *J. Agr. Food Chem., 2,* 1174 (1954).
170. Gardiner, J. E., and Kilby, B. A., *Biochem. J., 51,* 78 (1952).
171. Gardner, K., and Heath, D. F., *Anal. Chem., 25,* 1849 (1953).
172. Gardocki, J. F., and Hazleton, L. W., *J. Am. Pharm. Assoc., Sci. Ed., 40,* 491 (1951).
173. Garhardt, M. D., Witmer, F. J., and Tajima, Y. A., *Anal. Chem., 24,* 851 (1952).
174. Gehrke, C. W., Bevirt, J. L., and Johnston, M. R., *J. Agr. Food Chem.,* in press.
175. Gettler, A. O., and Sunshine, I., *Anal. Chem., 23,* 779 (1951).
176. Giang, P. A., private communication.
177. Giang, P. A., Barthel, W. F., and Hall, S. A., *J. Agr. Food Chem., 2,* 1281 (1954).
178. Giang, P. A., and Hall, S. A., *Anal. Chem., 23,* 1830 (1951).
179. Giang, P. A., Smith, F. F., and Hall, S. A., *J. Agr. Food Chem., 4,* 621 (1956).
180. Gjullin, C. M., Scudder, H. I., and Erwin, W. R., *J. Agr. Food Chem., 3,* 508 (1955).
181. Glasser, R. F., Blenk, R. G., Dewey, J. E., Hilton, B. D., and Weiden, M. H. J., Entomological Society of America, Eastern Branch Meeting, Baltimore, Md., Nov. 1955.
182. Glick, D., *J. Gen. Physiol., 21,* 289 (1938).
183. Goodhue, L. D., *J. Assoc. Offic. Agr. Chemists, 19,* 118 (1936).
184. Gordon, H. T., *Anal. Chem., 24,* 857 (1952).

185. Gordon, N., and Beroza, M., *Anal. Chem.*, *24*, 1968 (1952).
186. Gottlieb, S., and Marsh, P. B., *Ind. Eng. Chem.*, *Anal. Ed.*, *18*, 16 (1946).
187. Green, N., and Schechter, M. S., *Anal. Chem.*, *27*, 1261 (1955).
188. Grob, D., *Ann. Internal Med.*, *32*, 1229 (1950).
189. Gross, C. R., and Smith, C. M., *J. Assoc. Offic. Agr. Chemists*, *17*, 336 (1934).
190. Gruch, W., *Naturwissenschaften*, *41*, 39 (1954).
191. Grummitt, O., Marsh, D., and Stearns, J. A., *Anal. Chem.*, *24*, 702 (1952).
192. Gunther, F. A., *Ind. Eng. Chem.*, *Anal. Ed.*, *17*, 149 (1945).
193. Gunther, F. A., Barkley, J. H., and Ewart, W. H., *J. Econ. Entomol.*, *47*, 1033 (1954).
194. Gunther, F. A., Beier, R. L., and LaDue, J. P., *Ind. Eng. Chem.*, *Anal. Ed.*, *15*, 574 (1943).
195. Gunther, F. A., and Blinn, R. C., *Advances in Chem. Ser.*, *No. 1*, 72 (1950).
196. Gunther, F. A., and Blinn, R. C., *Anal. Chem.*, *22*, 1450 (1950).
197. Gunther, F. A., and Blinn, R. C., *Chemical Analysis*, Vol. VI, *Analysis of Insecticides and Acaricides*, Interscience Publishers, Inc., New York, 1955.
198. Gunther, F. A., and Blinn, R. C., *J. Agr. Food Chem.*, *1*, 325 (1953).
199. Gunther, F. A., Blinn, R. C., Kolbezen, M. J., Barkley, J. H., Harris, W. D., and Simon, H. S., *Anal. Chem.*, *23*, 1835 (1951).
200. Gunther, F. A., and Jeppson, L. R., *J. Econ. Entomol.*, *47*, 1027 (1954).
201. Hall, S. A., Stohlmann, J. W., and Schechter, M. S., *Anal. Chem.*, *23*, 1866 (1951).
202. Haller, H. L., Bartlett, P. D., Drake, N. L., Newman, M. S., Eaker, C. M., Hayes, R. A., Kilmer, G. W., Magerlein, B., Mueller, G. P., Schneider, A., and Wheatley, W., *J. Am. Chem. Soc.*, *67*, 1591 (1945).
203. Hamblin, D. O., and Golz, H. H., *Cholinesterase Tests and Their Applicability in the Field*, American Cyanamid Co., Sept. 1953.
204. Hamblin, D. O., and Marchand, J. F., *Cholinesterase Tests and Their Applicability in the Field*, American Cyanamid Co., Mar. 1951.
205. Hamblin, D. O., and Marchand, J. F., *Ann. Internal Med.*, *36*, 50 (1952); *Am. Practitioner and Dig. Treatment*, *2*, 1 (1951).
206. Hancock, W., and Laws, E. Q., *Analyst*, *80*, 665 (1955).
207. Harris, H. J., *J. Agr. Food Chem.*, *3*, 939 (1955).
208. Harris, H. J., Geigy Co., *Tentative Ultraviolet Method for Analysis of Diazinon in Spray Residues* (1954).
209. Harris, T. H., *J. Assoc. Offic. Agr. Chemists*, *34*, 672 (1951).
210. Harris, T. H., *J. Assoc Offic. Agr. Chemists*, *35*, 376 (1952).
211. Hartley, G. S., address before 15th International Chemical Congress, New York, Sept. 1951.
212. Hartley, G. S., *Chemistry & Industry*, *1954*, 529.
213. Hartley, G. S., *World Crops*, *4*, 397 (1952).
214. Hartley, G. S., and Heath, D. F., *Nature*, *167*, 816 (1951).
215. Hartley, G. S., Heath, D. F., Hulme, J. M., Pound, D. W., and Whittaker, M., *J. Sci. Food Agr.*, *2*, 303 (1951).
216. Hartley, G. S., and Kilby, B. A., *Nature*, *166*, 784 (1950).
217. Haskins, W. T., *Anal. Chem.*, *23*, 1672 (1951).
218. Hazleton, L. W., *J. Agr. Food Chem.*, *3*, 312 (1955).

219. Hazleton, L. W., and Holland, E. G., *Advances in Chem. Ser., No. 1*, 31 (1950).
220. Heath, D., paper read at 3rd Internat. Congr. Crop Protection, Paris, 1952.
221. Heath, D. F., and Casapieri, P., *Trans. Faraday Soc., 47*, 1093 (1951).
222. Heath, D. F., Cleugh, J., Otter, I. K. H., and Park, P. O., *J. Agr. Food Chem., 4*, 230 (1956).
223. Heath, D. F., Lane, D. W. J., and Llewellyn, M., *J. Sci. Food Agr., 3*, 60, 69 (1952).
224. Heigl, J. J., Bell, M. F., and White, J. U., *Ind. Eng. Chem., Anal. Ed., 19*, 293 (1947).
225. Helmkamp, G. K., Gunther, F. A., Wolf, J. P., and Leonard, J. E., *J. Agr. Food Chem., 2*, 836 (1954).
226. Henglein, A., Schrader, G., and Mühlmann, R., *Z. anal. Chem., 141*, 276 (1954).
227. Hensel, J., Hewitt, A. E., Sheets, U. M., and Scott, R. C., Chemagro Corp., revised, 1956.
228. Herriot, R. M., *Science, 104*, 228 (1946).
229. Hestrin, S., *J. Biol. Chem., 180*, 249 (1949).
230. Higgons, D. J., and Kilbey, D. W., *Chemistry & Industry, 1954*, 1359.
231. Higgons, D. J., and Kilbey, D. W., *J. Sci. Food Agr., 6*, 441 (1955).
232. Hillenbrand, E. F., Sutherland, W. W., and Hogsett, J. N., *Anal. Chem., 23*, 626 (1951).
233. Hirt, R. C., and Gisclard, J. B., *Anal. Chem., 23*, 185 (1951).
234. Hopf, H. S., *Ann. Appl. Biol., 39*, 193 (1952); *41*, 248 (1954).
235. Hopkins, L., Abu Yaman, I. K., Carruth, L. A., *J. Econ. Entomol., 48*, 151 (1955).
236. Hornstein, I., *J. Agr. Food Chem., 5*, 37 (1957).
237. Hornstein, I., *Anal. Chem., 24*, 1036 (1952).
238. Hornstein, I., *J. Agr. Food Chem., 3*, 848 (1955).
239. Hornstein, I., Abstracts of Papers, presented at 129th meeting of American Chemical Society, Dallas, Texas, April 1956.
240. Hornstein, I., and Sullivan, W. N., *Anal. Chem., 25*, 496 (1953).
241. Houston, F. G., *Anal. Chem., 24*, 1831 (1952).
242. Howard, B. H., *Analyst, 72*, 427 (1947).
243. Hudy, J. A., and Dunn, C. L., *J. Agr. Food Chem., 5*, 351 (1957).
244. Iling, E. T., and Stephenson, W. H., *Analyst, 71*, 310 (1946).
245. Janovsky, J. V., *Ber., 24*, 971 (1891).
246. Janovsky, J. V., and Erb, L., *Ber., 19*, 2155 (1886).
247. Jansen, E. F., and Balls, A. K., *J. Biol. Chem., 194*, 721 (1952).
248. Jansen, E. F., Curl, A. L., and Balls, A. K., *J. Biol. Chem., 190*, 557 (1951).
249. Jansen, E. F., Nutting, M. D. F., and Balls, A. K., *J. Biol. Chem., 170*, 417 (1947); *175*, 975 (1948); *179*, 201 (1949).
250. Jansen, E. F., Nutting, M. D. F., Jang, R., and Balls, A. K., *J. Biol. Chem , 179*, 189 (1949); *185*, 209 (1950).
251. Jeffrey, R. N., and Eoff, W. H., *Anal. Chem., 27*, 1903 (1955).
252. Jennings, E. C., and Edwards, D. G., *Anal. Chem., 25*, 1179 (1953).

253. Jensen, J. A., Durham, W. F., and Pearce, G. W., *Arch. Ind. Hyg. and Occupational Med.*, *6*, 326 (1952).
254. Johnson, D. P., *J. Assoc. Offic. Agr. Chemists*, *38*, 153 (1955).
255. Jones, G. D. G., and Thomas, W. D. E., *Ann. Appl. Biol.*, *40*, 546 (1953).
256. Jones, H. A., *A Review of Methods for the Chemical Analysis of Rotenone-Bearing Plants*, U. S. Dept. of Agriculture Publication E-563. Processed, April 1942.
257. Jones, H. A., Ackermann, H. J., and Webster, M. E., *J. Assoc. Offic. Agr. Chemists*, *35*, 771 (1952).
258. Jones, L. R., and Riddick, J. A., *Anal. Chem.*, *23*, 349 (1951).
259. Jones, L. R., and Riddick, J. A., *Anal. Chem.*, *24*, 569 (1952).
260. Jones, L. R., and Riddick, J. A., *Anal. Chem.*, *24*, 1533 (1952).
261. Keller, H., *Naturwissenschaften*, *39*, 109 (1952).
262. Kennett, B. H., *J. Agr. Food Chem.*, *2*, 691 (1954).
263. Kenyon, W. C., *Anal. Chem.*, *24*, 1197 (1952).
264. Ketelaar, J. A. A., *Rec. trav. chim.*, *69*, 649 (1950).
265. Ketelaar, J. A. A., and Hellingman, J. E., *Anal. Chem.*, *23*, 646 (1951).
266. Kilby, B. A., *Chemistry & Industry*, *1953*, 856.
267. Kittleson, A. R., *Anal. Chem.*, *24*, 1173 (1952).
268. Klein, A. K., *J. Assoc. Offic. Agr. Chemists*, *32*, 351 (1949).
269. Klein, A. K., *J. Assoc. Offic. Agr. Chemists*, *33*, 594 (1950).
270. Klein, A. K., *J. Assoc. Offic. Agr. Chemists*, *36*, 589 (1953).
271. Knudsen, H. W., Meloche, V. W., and Juday, C., *Ind. Eng. Chem., Anal. Ed.*, *12*, 715 (1940).
272. Kok, G. C., and Walop, J. N., *Biochim. et Biophys. Acta*, *13*, 510 (1954).
273. Kolbezen, M. J., and Barkley, J. H., *J. Agr. Food Chem.*, *2*, 1278 (1954).
274. Kolthoff, I. M., and Noponen, G. E., *J. Am. Chem. Soc.*, *55*, 1448 (1933).
275. Kowalkowski, R. L., Kennedy, J. H., and Farrington, P. S., *Anal. Chem.*, *26*, 626 (1954).
276. Krauze, S., Przybylski, E., and Tworek, R., *Roczniki Pańśtwowego Zakladu Hig.*, *1*, 3 (1950); through *Chem. Abstracts*, *45*, 6788 (1951).
277. Kruse, J. M., and Mellon, M. G., *Anal. Chem.*, *25*, 446 (1953).
278. Kunze, F. M., *J. Assoc. Offic. Agr. Chemists*, *37*, 578 (1954).
279. Kutschinski, A. H., and Luce, E. N., *Anal. Chem.*, *24*, 1188 (1952).
280. Labat, J. A., *Bull. soc. chim. biol.*, *15*, 1344 (1933).
281. Laitinen, H. A., Jennings, W. P., and Parks, T. D., *Ind. Eng. Chem., Anal. Ed.*, *18*, 355 (1946).
282. Larson, P. S., and Haag, H. B., *Ind. Eng. Chem., Anal. Ed.*, *16*, 86 (1944).
283. LaClair, J. B., *Anal. Chem.*, *20*, 241 (1948).
284. Lesuk, A., U. S. Patent 2,475,792 (1949); U. S. Patent 2,475,793 (1949).
285. Levine, J., and Fischbach, H., *J. Am. Pharm. Assoc., Sci. Ed.*, *44*, 713 (1955).
286. Levitsky, M., and Lowen, W. K., *J. Assoc. Offic. Agr. Chemists*, *37*, 555 (1954).
287. Levy, L. W., and Estrada, R. E., *J. Agr. Food Chem.*, *2*, 629 (1954).
288. Lieben, J., Waldman, R. K., and Krause, L., *Arch. Ind. Hyg. and Occupational Med.*, *7*, 93 (1953); *6*, 491 (1952).
289. Liggett, L. M., *Anal. Chem.*, *26*, 748 (1954).

290. Limperos, G., and Ranta, K. E., *Science, 117*, 453 (1953).
291. Lingane, J. J., *Anal. Chem., 26*, 622 (1954).
292. Lord, K. A., *Ann. Appl. Biol., 43*, 192 (1955).
293. Lord, K. A., *Nature, 165*, 567 (1950).
294. Lord, K. A., and Potter, C., *Chemistry & Industry, 1954*, 1214.
295. Lord, K. A., and Potter, C., *J. Sci. Food Agr., 5*, 490 (1954).
296. Lowen, W. K., *Anal. Chem., 23*, 1846 (1951).
297. Lowen, W. K., *J. Assoc. Offic. Agr. Chemists, 36*, 484 (1953).
298. Lowen, W. K., and Baker, H. M., *Anal. Chem., 24*, 1475 (1952).
299. Lubatti, F., and Harrison, A., *J. Soc. Chem. Ind. (London), 63*, 140 (1944)
300. March, R. B., Metcalf, R. L., and Fukuto, T. R., *J. Agr. Food Chem., 2*, 732 (1954).
301. March, R. B., Metcalf, R. L., Fukuto, T. R., and Maxon, M. G., *J. Econ. Entomol., 48*, 355 (1955).
302. Markwood, L. N., *J. Assoc. Offic. Agr. Chemists, 22*, 427 (1939).
303. Markwood, L. N., *J. Assoc. Offic. Agr. Chemists, 23*, 792 (1940).
304. Markwood, L. N., *J. Assoc. Offic. Agr. Chemists, 23*, 800 (1940).
305. Marquardt, R. P., and Luce, E. N., *Anal. Chem., 23*, 1484 (1951).
306. Marquardt, R. P., and Luce, E. N., *J. Agr. Food Chem., 3*, 51 (1955).
307. Martin, H., *Can. Entomologist, 85*, 414 (1953).
308. Martin, H., *Guide to the Chemicals Used in Crop Protection*, 2nd ed., Canada Department of Agriculture, 1953; and Supplement 1, 1955.
309. Martin, J. T., and Batt, R. F., *Ann. Rept. Agr. and Hort. Research Sta., Long Ashton, Bristol, 1953*, 121 (published 1954).
310. Martin, J. T., and Pickard, J. A., *Ann. Rept. Agr. and Hort. Research Sta., Long Ashton, Bristol, 1954*, 83 (published *1955*).
311. Mattson, A. M., Spillane, J. T., Baker, C., and Pearce, G. W., *Anal. Chem., 25*, 1065 (1953).
312. McCallan, S. E. A., Miller, L. P., and Magill, M. A., *Phytopathology, 45*, 295 (1955).
313. Mellon, M. G., *Analytical Absorption Spectroscopy*, John Wiley and Sons, Inc., New York, 1950.
314. Merck and Co., *Colorimetric Assay for Streptomycin*, 1955.
315. Metcalf, R. L., *Agr. Chemicals, 9*, No. 3, 33 (1954).
316. Metcalf, R. L., *J. Econ. Entomol., 44*, 883 (1951).
317. Metcalf, R. L., *Organic Insecticides*, Interscience Publishers, Inc., New York, 1955.
318. Metcalf, R. L., and March, R. B., *Ann. Entomol. Soc. Amer., 46*, 63 (1953).
319. Metcalf, R. L., and March, R. B., *J. Econ. Entomol., 42*, 721 (1949).
320. Metcalf, R. L., and March, R. B., *J. Econ. Entomol., 43*, 670 (1950).
321. Metcalf, R. L., and March, R. B., *J. Econ. Entomol., 45*, 988 (1952).
322. Metcalf, R. L., and March, R. B., *J. Econ. Entomol., 46*, 288 (1953).
323. Metcalf, R. L., and March, R. B., *Science, 117*, 527 (1953).
324. Metcalf, R. L., March, R. B., Fukuto, T. R., and Maxon, M., *J. Econ. Entomol., 47*, 1045 (1954).
325. Metcalf, R. L., March, R. B., Fukuto, T. R., and Maxon, M. G., *J. Econ. Entomol., 48*, 364 (1955).

326. Metcalf, R. L., Fukuto, T. R., Reynolds, H. T., and March, R. B., *J. Agr. Food Chem.*, *3*, 1011 (1955).
327. Michel, H. O., *J. Lab. Clin. Med.*, *34*, 1564 (1949).
328. Miller, V. L., and Polley, D., *Anal. Chem.*, *26*, 1333 (1954).
329. Miller, V. L., Polley, D., and Gould, C. J., *Anal. Chem.*, *23*, 1286 (1951).
330. Mitchell, L. C., *J. Assoc. Offic. Agr. Chemists*, *35*, 920, 928 (1952); *36*, 1183 (1953); *37*, 216, 530, 996 (1954).
331. Mitchell, L. C., and Patterson, W. I., *J. Assoc. Offic. Agr. Chemists*, *36*, 553 (1953).
332. Monsanto Chemical Co., Techn. Bull. 0–24, March 1955.
333. Monsanto Chemical Co., private communication.
334. Moore, B. P., *J. Sci. Food Agr.*, *5*, 500 (1954).
335. Morehead, F. F., *Ind. Eng. Chem., Anal. Ed.*, *12*, 373 (1940).
336. Morton, R. A., and Stubbs, A. L., *Analyst*, *71*, 348 (1946); *Biochem. J.*, *41*, 525 (1947); *42*, 195 (1948).
337. Moynihan, P., and O'Colla, P., *Chemistry & Industry*, *1951*, 407.
338. Munakata, K., *Japan Analyst*, *1*, 199 (1952).
339. National Academy of Sciences-National Research Council, Washington, D. C., *Annotated Bibliography of Analytical Methods for Pesticides*, Section I (1952); Section II (1954).
340. Neal, P. A., Von Oettingen, W. F., Smith, W. W., Malmo, R. B., Dunn, R. C., Moran, H. E., Sweeney, T. R., Armstrong, T. W., and White, W. C., *U. S. Public Health Repts., Suppl. No. 177*, p. 2 (1944).
341. Neal, P. A., Sweeney, T. R., Spicer, S. S., and Von Oettigen, W. F., *U. S. Public Health Repts.*, *61*, 403 (1946).
342. Norris, M. V., Vail, W. A., and Averell, P. R., *J. Agr. Food Chem.*, *2*, 570 (1954).
343. Norton, L. B., and Schmalzreidt, B., *Anal. Chem.*, *22*, 1451 (1950).
344. O'Brien, R. D., and Spencer, E. Y., *J. Agr. Food Chem.*, *3*, 56 (1955); *1*, 946 (1953).
345. O'Colla, P., *J. Sci. Food Agr.*, *3*, 130 (1952).
346. O'Donnell, A. E., Johnson, H. W., and Weiss, F. T., *J. Agr. Food Chem.*, *3*, 757 (1955).
347. O'Donnell, A. E., Neal, M. M., Weiss, F. T., Bann, J. M., Decino, T. J., and Lau, S. C., *J. Agr. Food Chem.*, *2*, 573 (1954).
348. Ogg, C. L., Willits, C. O., and Ricciuti, C., *Anal. Chem.*, *22*, 335 (1950).
349. Ohno, M., and Oiwa, T., *Bull. Inst. Chem. Research, Kyoto Univ.*, *31*, 389 (1953).
350. Oiwa, T., Inoue, Y., Mayeda, J., and Ohno, M., *Botyu-Kagaku*, *17*, 106 (1952).
351. Ordas, E. O., Smith, V. C., Meyer, C. F., *J. Agr. Food Chem.*, *4*, 444 (1956).
352. Oser, B. L., *Anal. Chem.*, *21*, 529 (1949).
353. Palumbio, M., *Farm. Sci. e tec. (Pavia)* *4*, 531 (1949).
354. Pankaskie, J. E., Fountaine, F. C., and Dahm, P. A., *J. Econ. Entomol.*, *45*, 51 (1952).
355. Parker, V. H., *Analyst*, *74*, 646 (1949).

356. Patchett, G. G., Stauffer Chemical Co., Richmond Research Lab., Feb 1955.
357. Pearce, G. W., Mattson, A. M., and Hayes, W. J., *Science, 116,* 254 (1952).
358. Peters, E. D., Rounds, G. C., and Agazzi, E. J., *Anal. Chem., 24,* 710 (1952).
359. Peterson, G. W., and Radke, H. H., *Ind. Eng. Chem., Anal. Ed., 16,* 63 (1944).
360. Petrino, L. M., Stauffer Chemical Co., Richmond Research Laboratory, March 1955.
361. Phillips, G. L., and Bulger, J. W., *U. S. Dept. Agr., Bur. Entomol. Plant Quarantine E-851* (1953).
362. Phillips, W. F., *Anal. Chem., 24,* 1976 (1952).
363. Phillips, W. F., and DeBenedictis, M. E., *J. Agr. Food Chem., 2,* 1226 (1954).
364. Pittsburgh Coke and Chemical Co., *Microestimation of B-622 Residues,* Research Development Dept. Report #171-1003, Jan. 1956.
365. Polen, P. B., and Silverman, P., *Anal. Chem., 24,* 733 (1952).
366. Polley, D., and Miller, V. L., *Anal. Chem., 24,* 1622 (1952).
367. Polley, D., and Miller, V. L., *Anal. Chem., 27,* 1162 (1955).
368. Polley, D., and Miller, V. L., *J. Agr. Food Chem., 2,* 1030 (1954).
369. Porter, C. C., *Anal. Chem., 27,* 805 (1955).
370. Prescott, G. C., Emerson, H., and Ford, J. H., *J. Agr. Food Chem., 4,* 343 (1956).
371. Prudhomme, R. O., *J. pharm. chim., 1* [9], 8 (1940); *Bull. soc. pathol. exotique, 31,* 929 (1938).
372. Radomski, J. L., and Davidow, B., *J. Pharmacol. Exptl. Therap., 107,* 266 (1953).
373. Ralston, W., *Anal. Chem., 10,* 533 (1938).
374. Ramamoorthy, B., Chatterjee, B. G., Dakshinamurti, C., and Gulati, K. C., *Nature, 169,* 112 (1952).
375. Ramos, V. P., de Carvalho, I., Neto, M. L., and Cabaco, E., *Oléagineux, 10,* 509 (1955); through *Chem. Abstracts, 49,* 15263 (1955).
376. Reith, J. F., *Chem. Weekblad, 49,* 689 (1953).
377. Reynolds, H. T., *Agr. Chemicals, 9,* No. 7, 28 (1954).
378. Rider, J. A., Moeller, H. C., and DuBois, K. P., *Proc. Soc. Exptl. Biol. Med., 76,* 427 (1951).
379. Rinthakul, C., and Hannen, J., *J. Soc. Chem. Ind. (London), 69,* 126 (1950).
380. Ripper, W. E., Greenslade, R. M., and Hartley, G. S., *Bull. Entomol. Research, 40,* 481 (1950).
381. Ripper, W. E., Greenslade, R. M., and Lickerish, L. A., *Nature, 163,* 787 (1949).
382. Robinson, R. H., *Anal. Chem., 27,* 1351 (1955).
383. Rohm and Haas Co., *Procedures for the Microdetermination of the Acaracide, Kelthane, Test Method No. L 13-2-1,* Nov. 1955.
384. Rohm and Haas Co., *Method for Karathane,* Jan. 1955.
385. Rosenthal, I., Carlsen, R. L., Stanley, E. L., *J. Assoc. Offic. Agr. Chemists, 36,* 1170 (1953).
386. von Rümker, R., *Agr. Chemicals, 10,* No. 1, 47 (1955).
387. St. John, J. L., *Anal. Chem., 25,* 42 (1953); *27,* 654 (1955).

388. Samuel, B. L., *J. Assoc. Offic. Agr. Chemists, 35,* 391 (1952).
389. Sandell, E. B., *Colorimetric Determination of Traces of Metals,* 2nd Ed., Interscience Publishers, New York-London, 1950.
390. S. B. Penick and Co., unpublished.
391. Schechter, M. S., and Haller, H. L., *J. Am. Chem. Soc., 66,* 2129 (1944).
392. Schechter, M. S., and Hornstein, I., *Anal. Chem., 24,* 544 (1952).
393. Schechter, M. S., LaForge, F. B., Zimmerli, A., and Thomas, J. M., *J. Am. Chem. Soc., 73,* 3541 (1951).
394. Schechter, M. S., Pogorelskin, M. A., and Haller, H. L., *Ind. Eng. Chem., Anal. Ed., 19,* 51 (1947).
395. Schechter, M. S., Soloway, S. B., Hayes, R. A., and Haller, H. L., *Ind. Eng. Chem., Anal. Ed., 17,* 704 (1945).
396. Schrenk, H. H., Pearce, S. J., and Yant, Y. P., *U. S. Bur. Mines, Rept. Invest.* 3287 (1935, revised 1937).
397. Schreiber, A. A., and McClellan, D. B., *Anal. Chem., 26,* 604 (1954).
398. Scott, E. W., and Treon, J. F., *Ind. Eng. Chem., Anal. Ed., 12,* 189 (1940).
399. Shell Development Company, Agricultural Research Division, *Photometric Determination of Endrin in Agricultural Crops,* Bull. ARMS-C-10/55.
400. Shell Development Company, Agricultural Research Division, *Photometric Determination of Isodrin Residues,* Bull. ARMS-C-7/54.
401. Shriner, R. L., and Fuson, R. C., *Identification of Organic Compounds,* 2nd Ed., p. 62, John Wiley & Sons, New York, N. Y., 1940.
402. Shukis, A. J., Cristi, D., and Wachs, H., *Soap Sanit. Chemicals, 27,* No. 11, 124 (1951).
403. Sinclair, W. B., and Crandall, P. R., *J. Econ. Entomol., 45,* 80 (1952).
404. Sinclair, W. B., and Crandall, P. R., *J. Econ. Entomol., 45,* 882 (1952).
405. Sinclair, W. B., and Ramsey, R. C., *Hilgardia, 16,* 291 (1944).
406. Smith, A. E., and Stone, G. M., *Anal. Chem., 25,* 1397 (1953).
407. Smith, F. F., Giang, P., and Fulton, R. A., *J. Econ. Entomol., 47,* 183 (1954).
408. Smith, F. F., Giang, P., and Taylor, E. A., *J. Econ. Entomol., 48,* 209 (1955).
409. Snell, F. D., and Snell, C. T., *Colorimetric Methods of Analysis,* 3rd Ed., Vol. III, D. Van Nostrand Co., Inc., New York, 1953.
410. Snell, F. D., and Snell, C. T., *Colorimetric Methods of Analysis,* 3rd Ed., Vol. IV, D. Van Nostrand Co., Inc., New York, 1954.
411. Spies, J. R., *J. Am. Chem. Soc., 58,* 2386 (1936).
412. Spies, J. R., *Ind. Eng. Chem., Anal. Ed., 9,* 46 (1937).
413. Spillane, J. T., and Pearce, G. W., presented at the 124th Meeting of the American Chemical Society, Chicago, Ill., Sept. 1953.
414. Stanley, E. L., Rosenthal, I., and Schmittinger, E. H., Philadelphia Meeting in Miniature, American Chemical Society, Feb. 1956.
415. Stanley, E. L., and Jackson, F., *Chem. Specialties Mfrs. Assoc., Proc.,* p. 104 (1954).
416. Stauffer Chemical Co., *Colorimetric Method for the Determination of R-1308 in Plant Materials.*
417. Stegwee, D., *Biochim. et Biophys. Acta, 8,* 187 (1952).
418. Stepanow, A., *Ber., 39,* 4056 (1906).
419. Stiff, H. A., and Castillo, J. C., *Science, 101,* 440 (1945).

420. Stiff, H. A., and Castillo, J. C., *J. Biol. Chem.*, *159*, 545 (1945).
421. Stohlman, E. F., and Smith, M. I., *J. Pharmacol. Exptl. Therap.*, *84*, 375 (1945).
422. Stone, C., Naugatuck Chemical Division of the U. S. Rubber Co., private communication.
423. Stroud, S. W., *Analyst*, *77*, 63 (1952).
424. Subba Rao, N. V., and Pollard, A. G., *J. Sci. Food Agr.*, *1*, 367 (1950).
425. Sund, K. A., *J. Agr. Food Chem.*, *4*, 57 (1956).
426. Suter, R., Delley, R., and Meyer, R., *Z. anal. Chem.*, *147*, 173 (1955).
427. Sutherland, G. L., Daroga, R. P., and Pollard, A. G., *J. Soc. Chem. Ind. (London)*, *58*, 284 (1939).
428. Tibbitts, T. W., and Holm, L. G., *J. Agr. Food Chem.*, *1*, 724 (1953).
429. Tomkins, R. G., and Isherwood, F. A., *Analyst*, *70*, 330 (1945).
430. Torda, C., and Wolff, H. G., *J. Pharmacol. Exptl. Therap.*, *95*, 444 (1949).
431. Trim, A. R., *Biochem. J.*, *43*, 57 (1948).
432. Tso, T. G., and Jeffrey, R. N., *Arch. Biochem. and Biophys.*, *43*, 269 (1953).
433. Tsuyuki, H., Stahmann, M. A., and Casida, J. E., *J. Agr. Food Chem.*, *3*, 922 (1955).
434. Umhoefer, R. R., *Ind. Eng. Chem., Anal. Ed.*, *15*, 383 (1943).
435. U. S. Department of Agriculture, *Yearbook of Agriculture, Insects*, pp. 302–316, 1952.
436. U. S. Public Health Service, Technical Development Branch, Communicable Disease Center, Savannah, Ga., Chem. Memo. 1, 1st revision, Sept. 1953.
437. U. S. Public Health Service, Technical Development Branch, Communicable Disease Center, Savannah, Ga., Chem. Memo. 2, June 1953.
438. United States Rubber Co., Naugatuck Chemical Division, Naugatuck, Conn., *Modifications of Aramite Method*, Feb. 1956.
439. Viles, F. J., *J. Ind. Hyg. Toxicol.*, *22*, 188 (1940).
440. Waldman, R. K., Lieben, J., and Krause, L., *Arch. Ind. Hyg. and Occupational Med.*, *9*, 37 (1954).
441. Walker, K. C., Goette, M. B., and Batchelor, G. S., *J. Agr. Food Chem.*, *2*, 1034 (1954).
442. Wallin, R. G., *Anal. Chem.*, *22*, 1208 (1950).
443. Warshowsky, B., and Schantz, E. J., *Anal. Chem.*, *22*, 460 (1950).
444. Weber, E., *Z. anal. Chem.*, *134*, 32 (1951).
445. Webster, J. G., and Dawson, J. A., *Analyst*, *77*, 203 (1952).
446. Westlake, W. E., and Butler, L. I., *J. Econ. Entomol.*, *46*, 850 (1953).
447. White, T. T., Penther, C. J., Tait, P. C., and Brooks, F. R., *Anal. Chem.*, *25*, 1664 (1953).
448. Williams, E. F., *Ind. Eng. Chem.*, *43*, 950 (1951).
449. Williams, H. L., Dale, W. E., and Sweeney, J. P., *J. Assoc. Offic. Agr. Chemists*, *39*, 872 (1956).
450. Williams, H. L., and Sweeney, J. P., *J. Assoc. Offic. Agr. Chemists*, *39*, 975 (1956).
451. Wilson, C. W., Baier, R., Genung, D., and Mullowney, J., *Anal. Chem.*, *23*, 1487 (1951).
452. Winteringham, F. P. W., *Science*, *116*, 452 (1952).

453. Willits, C. O., Swain, M. L., Connelly, J. A., and Brice, B. A., *Anal. Chem.*, *22*, 430 (1950).
454. Wolff, W. A., Hawkins, M. A., and Giles, W. E., *J. Biol. Chem.*, *175*, 825 (1948).
455. Wollenberg, O., and Schrader, G., *Angew. Chem.*, *68*, 41 (1956).
456. Wood, P. C., Naugatuck Chemical Division, United States Rubber Co., Naugatuck, Conn., Dec. 1952.
457. Wood, P. R., *Anal. Chem.*, *25*, 1879 (1953).
458. Wright, N., *Ind. Eng. Chem., Anal. Ed.*, *13*, 1 (1941).
459. Yamada, R., Sato, T., and Iwata, J., *Botyu-Kagaku*, *17*, 31 (1952).
460. Young, H. Y., and Gortner, W. A., *Anal. Chem.*, *25*, 800 (1953).
461. Zeumer, H., and Fischer, W., *Z. anal. Chem.*, *135*, 401 (1952).
462. Zeumer, H., and Neuhaus, K., *Chem.-Ztg.*, *77*, 105 (1953).

# BIOASSAY OF PESTICIDE RESIDUES

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## I. Introduction

The widespread use of synthetic organic pesticides and the high mammalian toxicity of many of them increase the importance of determining microquantities of pesticide residues in plant and animal tissues. Chemical analysis has been recognized as a standard method for analyzing pesticide residues. However, in the early development stage, such a specific method may not be available (14,18,38,44,58, 60,78,103), may not be sensitive enough (14,44,68,103,107), or may be interfered with by extractives (58,103). Even with adequate chemical methods, some laboratories may not be equipped to conduct certain chemical analyses. Bioassay may also be used as a supplementary method to check chemical analysis, and vice versa (5,11, 18,22,40,53,67). Furthermore, the development of bioassay methods gives many biologists the opportunity to do their own assays. Because of these reasons bioassays of insecticide residues have been rapidly developed and accepted not only by entomologists but also by chemists and other research workers. However, little work has been reported on the bioassay of other pesticide residues.

The main disadvantage of bioassay methods is the lack of specificity. In the bioassay of pesticide residues any chemical which is toxic to the test organism will interfere with the assay of another chemical. However, when a single stable toxicant is used, bioassay should give the same result as chemical analysis. On the other hand, the non-specific nature of bioassay results in a high degree of adaptability. Residues of various toxicants in different plant and animal tissues can be determined with only minor modifications of the basic procedures. The simplicity and adaptability of bioassay methods have won their acceptance in the field of residue analysis. The extreme sensitivity of some test organisms, such as mosquito larvae and Drosophila, is a great advantage in assaying minute quantities of certain toxicants.

In the development of bioassay methods, it appears that there are a number of similarities in principle and practice between bioassay and chemical analysis as well as between analytical bioassay of pesticide residues and bioassay of compounds as pesticides. The adoption of chemical techniques will immeasurably assist the further advancement of bioassay methods. At the same time, proper use of testing techniques in studying pesticides will give us more means and better techniques in our future bioassay of residues.

## II. Principle of Bioassay

The principle of bioassay is not new, although it has only recently been introduced in the assay of pesticide residues. Bioassay techniques have been well developed and widely used in pharmacy for testing the potency of vitamins and other drugs. Recently, Finney (37) gave a comprehensive account of designs and statistical analyses for these assays. Like the bioassay of vitamins and drugs, the determination of pesticide residues is based on the measurement of death, growth or other physiological responses of animals, plants or microorganisms. The major difficulty in the bioassay of pesticide residues is the isolation of extremely small quantities of toxicants in the presence of large amounts of plant or animal materials. Chemical analysis shares this same problem. Gunther and Blinn (47) reviewed the basic principles for the quantitative determination of pesticide residues. They emphasized that a thorough study of the chemical and physical properties of a pesticide is the first consideration in devising an analytical method for its determination on and in plants and in soils.

As is well known, the response of organisms varies from time to time. To reduce such variations it has been suggested that a standard toxicant be included in each series of tests. Rideal and Walker (91) compared the toxicity of bactericides by means of the phenol coefficient, which is the ratio of the killing dose of phenol to that of the unknown. Later, Young and Cooper (112) modified their procedure and used copper sulfate as a standard to determine fungicidal coefficients for the common fungicides. Fleming and Baker (39) determined the coefficient of effectiveness of a material by dividing the median lethal concentration of the standard insecticide by the median lethal concentration of the material under test. Bliss (9) measured the degree of agreement between different series of dosage-mortality data by reference to a standard curve. Wilcoxon and McCallan (111) suggested the use of a standard fungicide, such as bordeaux mixture, to compare the relative toxicity of fungicides. Horsfall *et al.* (56) used bordeaux mixture as a standard to compare fungicidal activities. There has been no serious consideration of the consistency of such expressions in relation to various factors affecting toxicity results. More recently, Sun (100) studied a number of factors affecting mortality and $LD_{50}$'s of insecticides and found that variations of $LD_{50}$'s of several insecticides sprayed on house flies did not appreciably affect the toxicity index, which is the ratio of the $LD_{50}$ of a standard and that of a test sample. The consistency of the toxicity index under different test conditions appears to arise from the fact that the change in the $LD_{50}$ of a test sample is accompanied by a proportional change in the $LD_{50}$ of the standard insecticide. He also pointed out that, theoretically, by using a standard having the same composition as the test sample, the results should be the same, when tested with any species of insect, by any proper method of applying insecticides, and under any testing conditions.

Sun and Sun (103) extended the concept of the toxicity index to the bioassay of aldrin and dieldrin residues. According to their findings, the mortality of insects should be the same if two extracts contain the same quantity of the same toxicant as well as the same amount of the same extracted materials. Therefore, standards should be made by adding suitable amounts of the same toxicant to an untreated extract prepared in the same way as the treated samples. If the extract from a treated sample gives the same mortality as one of the standards, the amount of insecticide residue in the treated sample should be the same as that in the standard. The presence of extracted substances, other than toxicants, should not change the results, but would decrease the sensitivity. For example, the aldrin content of a prepared rat diet containing 5.0 p.p.m. of aldrin was determined by the bioassay method. Even when the weight of total extractives was as high as 120 mg. per ml. and the $LD_{50}$ of aldrin in the rat-diet extract was about six times higher than aldrin alone, the amount of aldrin determined by the dry-film method (103) was 5.1 p.p.m. The same principle for compensating the effect of extractives was also used by Nolan and Wilcoxon (79), and was mentioned by other authors (10,32,53,58).

Hoskins and coworkers (32,58,59,60) strived to remove all extractives from the extract so that the isolated toxicant would be comparable to a standard prepared from the pure chemical. Theoretically, this approach should give the same results as that suggested by Sun and Sun (103) as long as the "complete" removal of extractives can be accomplished.

## III. Validity of Bioassay Methods

Although methods for bioassaying pesticide residues have generally been recognized as being acceptable, some workers may be skeptical of their validity as analytical tools. It is true that not all bioassay methods have been thoroughly studied. To establish the validity of a bioassay method it is necessary to assay a series of recovery samples prepared by adding several practical levels of toxicant to the untreated material. The added toxicant in the recovery sample should be carried through the entire procedure. For each toxicant in different materials similar recovery samples should be carried out before the bioassay of actual samples. In addition, comparative analyses should be performed by a standard chemical or bioassay method on some field-treated samples in order to detect any unusual problems. Unfortunately, some bioassay methods were not developed on this basis, and too often, later bioassays of various treated materials were based upon insufficient recovery data. However, substantial improvement has been made in later methods.

### A. RECOVERIES

In recovery studies, treatment of a material with known amounts of insecticides in a manner which would duplicate the natural situation is practically impossible, especially if the toxicant is systemic in action. The best possible approach is to intimately mix the added toxicant with the finely divided material and to process the mixture as if it were a field-treated sample.

Pagan and Hageman (84) obtained 88.6% and 100% average recovery of DDT added to beans and tomatoes, respectively, by testing the aqueous suspension of DDT acetone extract with guppies, *Lebistes reticulatus* Peters. Bushland (14) reported good recoveries when three butter samples containing 25 p.p.m. of lindane were extracted, treated with acid, and bioassayed with *Aedes aegypti* (L.) larvae. By adding acetone solutions of DDT and dieldrin to homogenized potatoes and allowing them to stand for five hours before extraction, recoveries of approximately 80% were achieved by Terriere and Ingalsbe (104) with mosquito larvae.

Laug (67) presented a number of data on the recovery of DDT added to urine and several tissues at different levels. These results were recalculated in terms of percentage recoveries and standard deviations: 87% for urine, 78% for blood, 104% for muscle, 107 ± 20% for spleen, 90 ± 20% for kidney and 98 ± 35% for liver. Individual recovery varied from 48 to 170%. Sun and Sun (98) found that by using a direct feeding method the recovery of dieldrin in milk was 0.115 ± 0.047 p.p.m. at 0.1 p.p.m., and 0.32 and 0.26 p.p.m. at 0.3 p.p.m. The variation was larger at the lowest limit of detection, i.e., 0.1 p.p.m. Lamb (65) stated that the variations of recovery tests on dieldrin and Perthane in tomatoes, and DDT and dieldrin in apples were within 20% of the amount added. The recoveries obtained by Tressler and Jenkins (107) were 87% and 70% for 10 p.p.m. of DDT and malathion added to spinach. Pfadt and Ryff (87) investigated the dieldrin residues in renal, omental fat, liver, flesh, and wool of sheep. They reported that at 0.1, 0.2, 0.3, or 0.6 p.p.m. the recovery was 70% or over. Klein *et al.* (63) showed that when 0.1 p.p.m. endrin was added to 100 g. of collards and carried through the entire procedure, a recovery of 70% was obtained. At the 0.5 p.p.m. endrin level, the recoveries ranged from 83–90%.

A summary of unpublished data (94) obtained by the dry-film method with house flies (103) and Drosophila (101) indicates that most recovery results are satisfactory. The average recovery of dieldrin was 104 ± 16.7% (S.D.) and 103 ± 17.0% at 0.1 and 0.3 p.p.m. in soil, and 87 ± 16.2% at 0.3 p.p.m. in corn. Isodrin recovery data showed 81 ± 5.7% and 102 ± 9.0% at 0.1 and 0.3 p.p.m. in carrots. The recovery of endrin was 91 ± 26.7% and 90 ± 7.7% at 0.1 and 0.3 p.p.m. in carrots; 99 ± 27.0% and 96 ± 17.7% at 0.1 and 0.3 p.p.m. in potatoes; and 119 ± 36.0% and 107 ± 18.0% at 0.1 and 0.3 p.p.m. in corn. At 5 p.p.m. the recovery of aldrin in rat diet was 101 ± 5.3%; at 200 p.p.m. a spray method (100) gave a recovery of 96 ± 5.9% of aldrin in rat diet. The average standard deviation from the mean for recovery samples was ±16.1% for bioassay, as compared to ±15.0% and ±16.5% for the same or similar materials analyzed by the colorimetric methods for aldrin (80) and dieldrin (81) at the same levels. The average recovery results are similar to those obtained by Laug (67). Large variations for some determinations may be due to unusual biological variation, interferences by extractives and possibly accidental contamination.

## B. BIOASSAY AND CHEMICAL ANALYSES

Satisfactory recovery data should indicate the validity of bioassay methods for residue analysis. However, the pesticide residues on and in treated crops may be more difficult to extract than the toxicants added to untreated materials. To further prove the validity of a new bioassay method it is desirable to analyze field-treated samples by both bioassay and a standard chemical method. It is also desirable to compare a new chemical method against a standard bioassay method. Table I shows representative data on the comparative assays by bioassay and other methods for the same materials treated under the same conditions.

Laug (67) analyzed DDT in rat fat and obtained good agreement between bioassay and colorimetric analyses. Nolan and Wilcoxon (79) found that colorimetric analysis of parathion residues in several crops agreed very well with those obtained by bioassay using mosquito larvae. At higher levels, the bioassay results of DDT and parathion in spinach, reported by Burchfield *et al.* (11), and dieldrin in butterfat and beef fat, obtained by Bann *et al.* (5), agreed with those obtained by chemical analysis. However, the average result by chemical analysis for heptachlor in seven crops was reported by Hartzell *et al.* (53) to be approximately 2.6 times higher than that obtained by bioassay using the photomigration technique. Such large variations may be due to low heptachlor residues in these crops; the extractives may have interfered with the bioassay and chemical analysis. In the same paper (53) a good agreement was obtained for chlordane residues on the same seven crops analyzed by bioassay and colorimetric methods. However, chlordane is a mixture containing *alpha*-chlordane, *beta*-chlordane and heptachlor, all of which give color reactions (24), but vary greatly in insect toxicity (73,100). The agreement between bioassay and chemical analyses of chlordane residues may not be a good example. In the study of absorption and translocation of insecticides through the root systems of plants, Starnes (97) reported that, by analyzing parathion in soil, potato plants and tubers, fairly good agreement was obtained by colorimetric analysis and by bioassay using *Aedes aegypti* larvae. Good agreement, in general, was found by Tressler and Jenkins (107) between results obtained by house fly bioassay and by chemical analyses for six insecticides in four fresh food products. Ward and Burt (108) noted that the agreement between colorimetric and biological results for the six figures obtained from leaf surfaces is reasonably good. The average value is 1.59 $\gamma$ per sq. cm. for colorimetric analysis and 1.62 $\gamma$ per sq. cm. for bioassay (Table I).

Carter, Nelson, and Gersdorff (18) pointed out that determination of organically bound chlorine was in general agreement with house fly tests of toxaphene residues from the sprayed alfalfa. Fleming *et al.* (40,43) found that there was no significant difference in the results obtained by chemical analysis of total chlorine or by bioassay of chlordane, DDT, TDE and toxaphene in soil using *Popillia japonica*

TABLE I
Comparison between Bioassay and Other Methods of Analyses of Treated Materials

| Treated material | Toxicant used | Bioassay | | Other analyses | | Reference |
|---|---|---|---|---|---|---|
| | | Method | Residue, p.p.m. | Method | Residue, p.p.m. | |
| Dock | DDT | Dry-deposit | 1.8$^{a}$ | Colorimetric | 2.4$^{a}$ | (108) |
| Marrow | DDT | Dry-deposit | 1.4$^{a}$ | Colorimetric | 1.2$^{a}$ | (108) |
| Cabbage | DDT | Dry-deposit | 2.0$^{a}$ | Colorimetric | 1.4$^{a}$ | (108) |
| Butterfat | Dieldrin | Dry-film | 95 | Colorimetric | 95 | (5) |
| Beef fat | Dieldrin | Dry-film | 86 | Colorimetric | 85 | (5) |
| Rat fat | DDT | Dry-film | 2.1$^{b}$ | Colorimetric | 2.5$^{b}$ | (67) |
| Plant extract$^{e}$ | Dieldrin | Dry-film | 213$^{c}$ | Spray | 204$^{c}$ | (94) |
| Spinach | Parathion | Dry-film | 6.8 | Spray | 6.8 | (107) |
| Spinach | Lindane | Dry-film | 9.9$^{b}$ | Spray | 5.4$^{b}$ | (107) |
| Spinach | DDT | Dry-film | 74 | Spray | 71 | (107) |
| Spinach | Malathion | Dry-film | 12 | Spray | 14 | (107) |
| Spinach | Chlordane | Dry-film | 0.2 | Total chlorine | 0.1 | (107) |
| Peaches | DDT | Dry-film | 8.3 | Colorimetric | 8.5 | (107) |
| Celery | Toxaphene | Dry-film | 3.6 | Total chlorine | 2.7 | (107) |
| Soil | Chlordane | Contact with soil | 3.17; 4.05$^{d}$ | Total chlorine | 3.59$^{d}$ | (40) |
| Soil | DDT | Contact with soil | 22.5; 19.0$^{d}$ | Total chlorine | 19.5$^{d}$ | (43) |
| Soil | TDE | Contact with soil | 10.5; —$^{d}$ | Total chlorine | 13.0$^{d}$ | (43) |
| Soil | Toxaphene | Contact with soil | 10.0; 13.5$^{d}$ | Total chlorine | 12.0$^{d}$ | (43) |
| Onion bulbs | Parathion | Aqueous suspension | 0.10 | Colorimetric | 0.11 | (79) |
| Potato foliage | Parathion | Aqueous suspension | 0.20 | Colorimetric | 0.30 | (79) |
| Corn foliage | Parathion | Aqueous suspension | 0.16 | Colorimetric | 0.16 | (79) |
| String beans | Parathion | Aqueous suspension | 0.83 | Colorimetric | 0.80 | (79) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Soil | Aldrin | Aqueous suspension | 3.0 | Total chlorine | 3.2 | (104) |
| Soil | Dieldrin | Aqueous suspension | 4.8 | Total chlorine | 5.5 | (104) |
| Soil | Chlordane | Aqueous suspension | 2.4 | Total chlorine | 2.8 | (104) |
| Soil | BHC | Aqueous suspension | 0.4 | Total chlorine | 2.0 | (104) |
| Soil | Toxaphene | Aqueous suspension | 2.1 | Total chlorine | 5.8 | (104) |
| Soil | DDT | Aqueous suspension | 2.1 | Total chlorine | 2.6 | (104) |
| Soil | Heptachlor | Aqueous suspension | 2.2 | Total chloride | 2.5 | (104) |
| Soil | Parathion | Aqueous suspension | 9.10 | Colorimetric | 12.13 | (97) |
| Potato plant | Parathion | Aqueous suspension | 0.064 | Colorimetric | 0.11 | (97) |
| Potato tubers | Parathion | Aqueous suspension | 0.15 | Colorimetric | 0.17 | (97) |
| Spinach | DDT | Photomigration | 35 | Chemical | 45 | (11) |
| Spinach | Parathion | Photomigration | 6.4 | Chemical | 6.0 | (11) |
| Potato | Heptachlor | Photomigration | 0.008 | Colorimetric | 0.021 | (53) |
| Yams | Heptachlor | Photomigration | 0.003 | Colorimetric | 0.026 | (53) |
| Cabbage | Heptachlor | Photomigration | 0.01 | Colorimetric | 0.012 | (53) |
| Turnip | Heptachlor | Photomigration | 0.006 | Colorimetric | 0.022 | (53) |
| Onion | Heptachlor | Photomigration | 0.006 | Colorimetric | 0.008 | (53) |
| Corn | Heptachlor | Photomigration | 0.01 | Colorimetric | 0.016 | (53) |
| Carrot | Heptachlor | Photomigration | 0.008 | Colorimetric | 0.03 | (53) |
| Spinach | Aldrin | Direct-feeding | 0.30 | Dry-film | 0.30 | (94) |
| Spinach | Dieldrin | Direct-feeding | 1.0 | Dry-film | 0.67 | (94) |
| Carrot | Aldrin | Direct-feeding | 0.36 | Dry-film | 0.30 | (94) |
| Carrot | Dieldrin | Direct-feeding | 0.83 | Dry-film | 0.97 | (94) |

[a] $\gamma$/sq. cm.

[b] Mg./g.

[c] $\gamma$

[d] Lb./acre.

[e] 200 $\gamma$ dieldrin added to plant extracts and submitted as an unknown.

Newm. or *Macrocentrus ancylivorus* Roh. Terriere and Ingalsbe (104) obtained good agreement between total chlorine analyses and bioassay with mosquito larvae for seven insecticide residues in soil, but not for BHC and toxaphene (Table I). Claborn and Wells (19) used the total chlorine method to analyze dieldrin in milk. The results agreed very closely with bioassay (direct feeding) data on milk samples from two other cows similarly treated. Terriere and Kiigemagi (105) reported that the total chlorine analyses and bioassay with mosquito larvae gave good correlation for assaying aldrin, dieldrin, and heptachlor residues in several crops and in soil, and isodrin and endrin residues in broccoli. The coefficient of correlation between the two methods is 0.948 for aldrin, dieldrin and heptachlor in soil.

The validity of a new bioassay method or technique may be compared with a standard one for analyzing pesticide residues, especially in the absence of a standard chemical method for comparison. Sun and Sun (103) demonstrated that, by adding a suitable amount of the same insecticide to samples, the mortality of insects increased to a proper range. Small sublethal quantities of an insecticide can be detected with a reasonable accuracy. Dieldrin was found to be 1.5, 0 8, 1.7, and 1 4 p p.m. in milk samples by direct determination, and 1.8, 0.6, 1.9, and 1.7 p.p.m., respectively, by adding a known amount of dieldrin to each sample. When the same samples were assayed both by a dry-film method (103) and by a direct feeding method (85), no substantial differences in aldrin or dieldrin were noticed in treated spinach or carrot. Two hundred micrograms of dieldrin in a plant extract, chromatographed for chemical analysis, were found to be 213 and 204 $\gamma$ by the dry-film method (103) and the spray method (100), respectively, using house flies (Table I).

## IV. Bioassay Methods

Unlike specific chemical analysis, a bioassay method can be adapted for the assay of many pesticide residues in a variety of materials with different test organisms. Methods for bioassaying insecticide residues can be grouped together according to the method of exposure.

### A. DRY-FILM METHODS

This includes a group of methods, by which the test organisms are exposed to a "dry-film" of toxicant obtained by evaporating a portion of sample extract to dryness. The toxicant may be deposited as crystals or in solution. Active insects, such as house flies and Drosophila have been used as test animals.

As a pioneer in the field of bioassaying insecticide residues Laug has developed a bioassay method for DDT (67) and γ-BHC (68) in animal tissues. The toxicant was deposited by the evaporation of the solvent (ether) from the standard solution or tissue extract. To prevent the loss of γ-BHC during and subsequent to the evaporation of the ether solution, 20 mg. of corn oil were added to each flask. By using this method, difficulties were noticed from "tissue toxicity" caused by extractives from liver and other organs (67,68) and from masking the toxicity of insecticide by excessive amounts of extractives (63,68).

Hoskins and Messenger (58) used a small vial whose entire inner surface was treated. They pointed out that the accessibility of the deposit to the house flies by contact is the chief feature of the small vial method. However, results obtained by Dahm and Pankaskie (22), Sun and Sun (103) and Sun and Pankaskie (101) on house flies and Drosophila showed that it was not necessary to cover all surfaces of the test jar with the extract. As long as the toxic residues were deposited in the same manner for both the standard and the treated samples, the final results did not vary appreciably. This may be due to the high activity of flies which can pick up toxicants which are not uniformly deposited on all surfaces. Instead of compensating for the effect of extractives (103), Hoskins and Messenger (58) removed the extractives from a γ-BHC chloroform extract of chicken flesh by acid treatment; they also separated the extractives from a parathion chloroform extract of cabbage by passage through an absorption column. It appears that the dosage-mortality curve of either extract approaches that of the standard containing the toxicant only. Hoskins and coworkers (32,58,59,60) have attempted to develop cleanup methods which will remove practically all extractives of various materials from different toxicants.

Before the publication of Hoskins and Messenger's (58) method, Furman and Hoskins (45) bioassayed the γ-isomer equivalent in cream from cows treated with benzene hexachloride. They found that there was a loss of toxicity when residues of an acetone or chloroform solution of pure isomer were heated at 70°C. for five minutes.

Comparative analyses of the same samples by the bioassay methods developed by Sun and Sun (103) and by Hoskins and Messenger (58) indicate that the latter method tends to give somewhat higher results for more volatile compounds, such as lindane and aldrin. Such differences may be explained by a slightly higher loss of volatile compounds in the standard containing no extractives. Even after a proper cleanup, the extractives in various samples may be different. Therefore, in many cases, it would appear to be necessary to compensate for these differences, according to the methods described by Sun and Sun (103).

The effect of extractives on bioassay results was also noticed and corrected for by other workers. In the bioassay of endrin residues in leafy vegetables, with the method developed by Laug (67), Klein *et al.* (63) reported that if more than 10 mg. of plant residue is present in each flask, marked inhibition of insecticidal action may result. In order to simulate a "residue," 5 mg. of oleic acid were added from a petroleum ether solution to each of the endrin standard flasks. Such compensation for the effect caused by plant extractives was also used earlier by Dahm and Pankaskie (22). However, the difference in the quantity and quality of oleic acid or corn oil, as compared to plant or animal extractives, may produce results quite different from those obtained by the addition of the same extractives to the standard (103).

Davidow and Laug (25) developed a "masking technique" for bioassaying lindane deposits on paper, painted surface, cellophane and linoleum. An aliquot of a surface is prepared for test by masking the remaining portion of the experimental area with Scotch tape. The method is based upon the principle that, within the limits of the assay, a quantity of insecticide distributed over a small area will result in the same percentage mortality to house flies as the identical quantity of insecticide spread over a larger area. Repeated assays result in the conclusion that the masking of portions of the total area is equivalent to taking aliquots of the dose within experimental variations.

## B. AQUEOUS SUSPENSION METHODS

High sensitivity has been obtained by exposing mosquito larvae, microcrustaceans, or fish in an aqueous suspension or solution of toxicant. Their high susceptibility is attributed to the circulation and absorption of toxicant through the movement of gills or equivalent organs. According to this type of assay, the entire body of the organism is exposed to the toxicants.

Nolan and Wilcoxon (79) have devised a bioassay method for insecticides in plant materials using *Aedes aegypti* larvae. This method involves the extraction of parathion with benzene, evaporation of the benzene from aliquot portions, resuspension of the residues in water and testing with mosquito larvae. Other mosquito larvae and modified testing methods were described by Hartzell and Storrs (52) and Terriere and Ingalsbe (104). A method (83) in which guppies are used as test animals for bioassaying derris and lonchocarpus roots was modified by Pagan and Hageman (84) to detect small amounts of DDT residues. On the basis of the same principle, daphnids (1,2,3, 64,109), brine shrimps (76,77), and fish (84) were used for detecting toxic residues.

Mortality is not the only criterion upon which the bioassay of toxicants is based. Other physiological responses may also be used as criteria for measuring the effect of insecticides in a shorter time interval. The gain in time is usually accompanied by a sacrifice of sensitivity. By observing the "locomotory paralysis" of confused flour beetles, *Tribolium confusum* Duv., Sun (99) determined, in a few minutes, the amounts of carbon tetrachloride, chloropicrin, carbon disulfide and ethyl formate at 1- and 8-inch depths in 6.4-liter fumigation flasks. Burchfield, Hilchey and Storrs (11) developed a photomigration method for bioassay using mosquito larvae. The larvae of *A. aegypti* are confined behind a porous barrier in a shallow glass trough containing a dilute aqueous suspension of insecticide. When a 500-watt light is turned on and the barrier removed, viable larvae rapidly migrate to the far end. After an exposure of one minute, a second barrier is dropped into place and the larvae that are trapped behind it are regarded as moribund. Results are computed relative to standard curves determined on larvae withdrawn from the same batch. This method secures results in an hour or less. Newman (78) commented that although the photomigration method is effective, it has not been found to be any quicker or more reliable than direct observation by experienced staff.

### C. FEEDING METHODS

In addition to the above contact methods, various feeding methods have been devised for the bioassay of pesticide residues with or without extraction. Frawley *et al.* (44) described a technique for the bioassay of insecticides by oral administration to house flies. The procedure consists of making an ether extract of the sample, evaporating it to dryness over sugar, dissolving the sugar in an aqueous medium and allowing the flies to feed *ad libitum* on the solution for 24 hours. This technique obviates the meticulous purification of sample extracts frequently necessary for other bioassay procedures.

Sun and Sun (98) published a bioassay method for the determination of dieldrin in milk by direct feeding of house flies with homogenized milk. The results are obtained, in parts per million of toxicant, by comparing the mortality of flies in treated samples against the mortality in standards which are prepared by adding known amounts of the same toxicant to an untreated milk. It is logical that this method can also be applied to juices and liquid food. The direct feeding method of macerated food reported by Pankaskie and Sun (85) and Sun and Pankaskie (101) is a further extension of the above feeding method to solid materials. This method consists essentially of exposing pomace flies, *Drosophila melanogaster* Meig., to macerated plant tissues. To determine the amount of insecticide residue on plants previously treated with insecticide, mortalities resulting from exposure to this plant material are compared to mortalities resulting from exposure to standards prepared from untreated plant material by adding known amounts of the same toxicant. This appears to be a simple method for routine bioassay, if the required sensitivity can be obtained.

## D. ENZYMATIC METHODS

Most organic phosphorus insecticides are highly toxic to mammals and are generally very active inhibitors of cholinesterase and certain other enzymes. The principle of the cholinesterase (ChE) method for detecting microquantities of phosphorus insecticides can be illustrated as follows:

$$\text{ChE} + \text{inhibitor} \longrightarrow \text{inhibited ChE} + \text{uninhibited ChE}$$

$$\text{Acetylcholine} + \text{uninhibited ChE} \longrightarrow \text{choline} + \text{acetic acid}$$

The change of pH or the quantity of acetylcholine not hydrolyzed can be used for the calculation of inhibitor. The details of enzymatic methods for assaying insecticide residues are reviewed in the chapter on Chemical Analysis of Pesticide Residues.

### E. OTHER METHODS

Recently Harris (50) reviewed the use of microorganisms as analytical tools. Assay methods were described for antibiotics, vitamins and amino acids. In the early development the standard error ranged about ±10 to 15%. More recently Lees and Tootill (70,71) have shown that with large plate and routine care a standard error of ±5% could be obtained. Since the use of some antibiotics has been recently studied for the control of plant diseases, it is possible that the serial dilution methods, diffusion methods (or cylinder method) and turbidimetric methods used for antibiotic assays (54,70,89) could be used for the assay of antibiotic or fungicide residues.

## V. Bioassay With Various Organisms

Theoretically any organism which is susceptible to a pesticide may be used for the bioassay of its residues. However, to obtain a sensitivity of 1.0 to 0.1 p.p.m. of a toxicant, only a few selected organisms can be used. In addition to the requirement of high sensitivity, ease of rearing and handling are also essential for routine analyses. Extractives may interfere differentially with various organisms and organisms may respond differently as the methods are varied. Therefore, proper selection of test organism and bioassay method must be made in order to obtain accurate results. The following review summarizes the uses of various organisms for bioassaying different pesticides in plant and animal materials. Sensitivity and reproducibility are given when available. The methods of calculating sensitivity are given in the section on calculation.

### A. HOUSE FLY

House flies, *Musca domestica* L., are probably the most available insects and are commonly used for the bioassay of insecticide residues. They are easy to rear in large numbers and easy to handle. At room temperature their life cycle requires about two weeks for completion and the emergence is uniform. Adult flies of both sexes have often been used. They are relatively unaffected by extractives or pesticides other than insecticides.

Laug (67,68) and Laug and Fitzhugh (69) used house flies to bioassay dry deposits of DDT and $\gamma$-BHC obtained from rabbit and rat tissue extracts. Approximately 2.5 p.p.m. of DDT (67) and 0.2 p.p.m. of $\gamma$-BHC can be determined in animal tissues other than fat

TABLE II
Sensitivity of Organisms Used in the Bioassay of Pesticides

| Organism | Bioassay method | Toxicant | $LD_{50}$ of toxicant | | Sensitivity[a], per test container, $\gamma$ | Reference |
|---|---|---|---|---|---|---|
| House fly | Oral administration | DDT | 15 | $\gamma$/g. | 7.5 | (44) |
| House fly | Oral administration | Parathion | 3 | $\gamma$/g. | 1.5 | (44) |
| House fly | Oral administration | EPN | 4 | $\gamma$/g. | 2.0 | (44) |
| House fly | Oral administration | Lindane | 5 | $\gamma$/g. | 2.5 | (44) |
| House fly, male | Dry-film | p,p'-DDT | 3.7 | $\gamma$/vial | 1.9 | (58) |
| House fly, female | Dry-film | p,p'-DDT | 6.7 | $\gamma$/vial | 3.4 | (58) |
| House fly, male | Dry-film | $\gamma$-BHC | 1.4 | $\gamma$/vial | 0.7 | (58) |
| House fly, female | Dry-film | $\gamma$-BHC | 2.7 | $\gamma$/vial | 1.4 | (58) |
| House fly, female | Dry-film | Parathion | 0.56 | $\gamma$/vial | 0.28 | (58) |
| House fly | Dry-film | $\gamma$-BHC[b] | 0.75 | $\gamma$/vial | 0.38 | (60) |
| House fly | Dry-film | $\gamma$-BHC[c] | 0.48 | $\gamma$/vial | 0.24 | (60) |
| House fly | Dry-film | $\gamma$-BHC | 4.0 | $\gamma$/jar | 0.80 | (101) |
| House fly | Dry-film | Aldrin | 1.2 | $\gamma$/jar | 0.23 | (101) |
| House fly | Dry-film | Dieldrin | 0.75 | $\gamma$/jar | 0.15 | (101) |
| House fly | Dry-film | DDT | 60 | $\gamma$/jar | 12 | (101) |
| House fly | Dry-film | Aldrin | 1.26 | $\gamma$/g. | 0.63 | (22) |
| House fly | Dry-film | Endrin | 2 | $\gamma$/g. | 1.0 | (63) |
| House fly | Dry-film | $\gamma$-BHC | 0.72 | $\gamma$/g. | 0.36 | (68) |
| House fly (15.3 mg.) | Dry-film | Dieldrin | 1.0 | $\gamma$/jar | 0.2 | (102) |
| House fly (5.5 mg.) | Dry-film | Dieldrin | 0.5 | $\gamma$/jar | 0.1 | (102) |

| | | | | | | |
|---|---|---|---|---|---|---|
| *Aedes aegypti* | Aqueous suspension | Parathion | 0.003 | p.p.m.; 15 ml. | 0.023 | (51) |
| *A. aegypti* | Aqueous suspension | Lindane | 0.1 | p.p.m.; 15 ml. | 0.75 | (51) |
| *A. aegypti* | Aqueous suspension | Lindane | — | | $0.12^d$ | (52) |
| *A. aegypti* | Aqueous suspension | Aldrin | — | | $0.04^d$ | (52) |
| *A. aegypti* | Aqueous suspension | Dieldrin | — | | $0.12^d$ | (52) |
| *A. aegypti* | Aqueous suspension | DDT | — | | $0.47^d$ | (52) |
| *A. aegypti* (3rd instar) | Aqueous suspension | Parathion | 0.5 | γ/50 ml. | 0.25 | (78) |
| *A. aegypti* (1st instar) | Aqueous suspension | Parathion | 0.002 | γ/1.5 ml. | 0.001 | (78) |
| *A. aegypti* | Photomigration | DDT | 0.00306 | p.p.m.; 100 ml. | 0.15 | (11) |
| *A. aegypti* | Photomigration | Methoxychlor | 0.0233 | p.p.m.; 100 ml. | 1.2 | (11) |
| *A. aegypti* | Photomigration | Parathion | 0.0028 | p.p.m.; 100 ml. | 0.14 | (11) |
| *Culex quinquefasciatus* | Aqueous suspension | Parathion | 0.005 | p.p.m.; 15 ml. | 0.038 | (51) |
| Pomace fly | Dry-film | γ-BHC | 0.45 | γ/vial | 0.09 | (101) |
| Pomace fly | Dry-film | Aldrin | 0.25 | γ/vial | 0.05 | (101) |
| Pomace fly | Dry-film | Dieldrin | 0.20 | γ/vial | 0.04 | (101) |
| Pomace fly | Dry-film | DDT | 10 | γ/vial | 2.0 | (101) |
| Pomace fly | Direct-feeding | Aldrin | — | | 0.1 p.p.m. | (85) |
| Pomace fly | Direct-feeding | Dieldrin | — | | 0.1 p.p.m. | (85) |
| Pomace fly | Direct-feeding | DDT | — | | 5 p.p.m. | (85) |
| Pomace fly | Direct-feeding | Aldrin | 0.20 | p.p.m. | 0.05 p.p.m. | (38) |
| Guppies | Aqueous suspension | DDT | 0.05 | p.p.m. | 0.025 p.p.m. | (84) |
| *Gammarus pulex* | Aqueous suspension | Parathion | 5 | γ | 2.5 | (15) |

[a] Based on author's test method and calculation, or one-half of $LD_{50}$'s.

[b] 60-minute exposure.

[c] 240-minute exposure.

[d] Calculated by Sun and Pankaskie (101).

(68). The average $LD_{50}$ of γ-BHC for the house fly was 0.72 γ per gram of flies. Using a similar bioassay method Dahm and Pankaskie (22) determined aldrin in fat, liver, bile, muscle, blood, brain, kidney, spleen, feces and milk of cows and on forage crops, such as alfalfa. The sensitivity of the method is reported as 1 γ of aldrin (Table II), but no results of analyses and no sensitivity for each crop were given. The same method was used by Kitselman *et al.* (62) for assaying aldrin in various tissues of sheep and cattle. Klein *et al.* (63) determined endrin in broccoli, collards, kale and peppers at the level of 0.1 and 0.5 p.p.m. with an average deviation of 0.027 p.p.m. in extracts containing 0.13 to 1.0 p.p.m. of endrin. The average percentage deviation from the mean varied from 0 to 100%, with an overall average of ±34.4% for broccoli, ±13.2% for kale and ±11.6% for peppers. Carter, Nelson and Gersdorff (18) used house flies for the detection of toxaphene residues on alfalfa hay as a qualitative check for total chlorine analysis.

Hoskins and Messenger (58) developed a bioassay method using small shell vials. The method is capable of detecting microquantities of DDT, γ-BHC and parathion in chicken flesh or cabbage. The $LD_{50}$'s are shown in Table II. Apparently, the same method was used by Hoskins (57) for the determination of benzene hexachloride residues, as the γ-isomer, in carrots, corn, potatoes, sugar beets, tomatoes and chicken tissues. The sensitivity was 0.1 p.p.m. of gamma equivalent. In most cases, results for duplicate samples agreed to within less than 25% in case of the larger residues and often within a few per cent. Agreement was poorer with the low residues. Furman and Hoskins (45) bioassayed the γ-isomer equivalent of benzene hexachloride in cream with house flies before the perfection of the above method (58). If 14% fly mortality is considered as a significant figure, only 2.7 p.p.m. of γ-isomer was detectable. Lamb (65) used the cleanup and bioassay methods developed by Hoskins and coworkers for assaying the residues of 14 insecticides in a number of vegetables, fruits, and animal tissues. Tressler and Jenkins (107) determined parathion, lindane, DDT, malathion, chlordane and toxaphene residues in spinach, beans, peaches, or celery, using the house fly bioassay method.

The dry-film method developed by Sun and Sun (103) can detect 0.4 γ of aldrin or dieldrin in each jar containing low extractives, or 0.1 ppm of aldrin or dieldrin in a 100 g. sample. This method was also applied to the assay of aldrin and dieldrin in animal tissues (5). Table II shows the sensitivity of this method to $\gamma$-BHC, aldrin, dieldrin and DDT (101). To date this method has been used for the determination of aldrin, dieldrin, isodrin or endrin in 59 types of plant materials and 13 types of animal tissues. Approximately 0.1 p.p.m. of the above toxicants can be detected with house flies or pomace flies in 100–300 g. of sample. To determine the effects of weathering and commercial dehydration upon insecticide residues in alfalfa, Dahm (20) presented aldrin residues with a sensitivity of 0.1 p.p.m. Pfadt and Ryff (87) reported that dieldrin contents in renal, omental fat, liver, flesh and wool of sheep were determined by the dry-film method with house flies. The sensitivity of the method was 0.1 p.p.m. of dieldrin. In the study of insecticidal control of lice on chickens, Fairchild and Dahm (35) assayed dieldrin or lindane in tissues by the bioassay methods of either Sun and Sun (103) for dieldrin or Hoskins and Messenger (58) for lindane.

The feeding method reported by Sun and Sun (98) can determine 0.1 p.p.m. of dieldrin or aldrin, 0.18 p.p.m. of lindane, 0.5 to 0.6 p.p.m. of isodrin or endrin and 1.4 p.p.m. of DDT in milk with young flies in 48 hours. Duplicate tests of the same sample gave 1.5 and 1.45 p.p.m. of dieldrin in milk. However, Carter *et al.* (17) could not detect 50 p.p.m. of DDT in synthetic cream. Frawley, Laug and Fitzhugh (44) fed the extracts, to house flies, of apples, cucumbers, strawberries, peas and yeast. The method is sensitive to 3 $\gamma$ of parathion, 4 $\gamma$ of EPN, 5 $\gamma$ of lindane and 15 $\gamma$ of DDT.

### B. POMACE FLY

Pomace flies, *Drosophila melanogaster* Meig., can be used in place of house flies for the bioassay of insecticide residues with essentially the same extracts and the same testing procedures (101). They can easily be reared on canned pumpkin containing a few drops of yeast suspension (7). Compared to house flies, Drosophila has a shorter life cycle, is easier to handle in large numbers, and needs less space. Adults, up to a few days old, can be used for bioassay. A culture of Drosophila can be obtained from most genetics departments. In the study of the direct feeding method (85), field collected cultures of several species of Drosophila were also tested; no large differences in susceptibility to aldrin or dieldrin were noticed.

Pomace flies were first used by Pankaskie and Sun (85) for testing insecticide residues in macerated food, and by Sun and Pankaskie (101) for assaying toxicants in extracts. By direct feeding 0.1 p.p.m of aldrin and dieldrin, and 5 p.p.m. DDT can be detected in many fresh and canned fruits and vegetables (85,101); however, the sensitivity is not quite as high for cereal grains or soil samples. Generally, Drosophila are more sensitive than house flies (101). However, by the direct feeding method, pomace flies are much less sensitive than house flies for the detection of aldrin, dieldrin, lindane, and DDT in milk (101). Fisher and Smallman (38) reported that residues of 0.36 ± 0.03 p.p.m. and 0.18 ± 0.02 p.p.m. of dieldrin in canned apple sauce were found by the direct feeding method with Drosophila. The valid estimation of aldrin in pumpkin may be as low as 0.05 p.p.m. In the study of residues on strawberries grown in insecticide-treated soil, Gyrisco *et al.* (49) concluded that bioassay with *D. melanogaster* by direct feeding on macerated fruit, similar to the method proposed by Olson (82), was the most sensitive and reliable. The sensitivity of this method is 0.1 p.p.m. for aldrin, dieldrin, and heptachlor and 0.2 p.p.m. for lindane.

By the dry-film method, as little as 0.05 $\gamma$ of aldrin or dieldrin per test jar can be detected (101). On the basis of the minimum detectable quantities of several toxicants, pomace flies are approximately as sensitive as mosquito larvae (Table II). Treon *et al.* (106) reported the assay of endrin in dog liver, kidney, and brain by the dry-film method using pomace flies.

### C. MOSQUITO LARVAE

It has been demonstrated that mosquito larvae are extremely sensitive to insecticides, and thus they are very useful in bioassaying microquantities of toxic residues. Eggs of some species can be kept viable for several months. However, they have longer life cycles and are not reared or handled as easily as house flies or pomace flies. Mosquito larvae are also more sensitive to natural constituents extracted from some materials.

Simmons (95) reported a simple bioassay method involving exposure of mosquitoes to the sprayed surface and comparison of mortality to that caused by known deposits of DDT.

Nolan and Wilcoxon (79) detected 0.02 p.p.m. of parathion in vegetables with *Aedes aegypti* larvae. Hartzell and Storrs (52) adapted the method to bioassay heptachlor, aldrin, dieldrin, chlordane, lindane, methoxychlor, TDE and toxaphene in processed foods, such as strained beans, and mixtures of apricots and apples. The breaking point ranges of several insecticides were reported. Both *A. aegypti* and the southern house mosquito larvae, *Culex quinquefasciatus* Say, were used by Hartzell (51) for detecting toxic residues in fresh fruits and vegetables that had been sprayed or dusted with parathion, DDT, DDD, basic lead arsenate, toxaphene, or TEPP. Attempts were made by Bushland (14) to use *A. aegypti* and *Anopheles quadrimaculatus* larvae for the bioassay of DDT, TDE, methoxychlor, lindane, chlordane and toxaphene in milk, butterfat or meat. The sensitivity of this method is approximately 0.5 p.p.m. of lindane in milk containing 4% butterfat; however, it is far less sensitive to toxaphene and chlordane. Larvae of *Aedes vexans*, *A. sticticus*, and *C. quinquefasciatus* were used by Terriere and Ingalsbe (104) to bioassay the residues of several insecticides in soil and in plants. This method can detect as low as 0.02 p.p.m. of aldrin, dieldrin, BHC, DDT, and heptachlor, 0.04 p.p.m. of toxaphene, 0.06 p.p.m. of chlordane, and 0.005 p.p.m. of parathion and EPN in the potato tissue. With *C. quinquefasciatus* larvae, Terriere and Kiigemagi (105) detected sub-microgram quantities of 16 out of 21 different insecticides. Parathion, malathion, EPN, DDT, lindane, Diazinon, methoxychlor, and TDE can be assayed without cleanup, if one is interested in determining whether or not a sample will meet the food and drug tolerance requirements. Newman (78) detected 0.02 p.p.m. of parathion in a 100 g. sample of material by bioassay using *A. aegypti* larvae. He also determined the parathion residues in soil and in tomatoes.

A photomigration method based on the negative phototaxic response of *A. aegypti* larvae was developed by Burchfield, Hilchey and Storrs (11) for bioassaying DDT, methoxychlor, lindane, parathion and aldrin residues on sprayed spinach. This method can detect very low concentrations of DDT and parathion (Table II). Hartzell, Storrs and Burchfield (53) used the above method for the bioassay of chlordane and heptachlor on treated potato, yams, cabbage, onion, carrot, corn and turnip. The results for heptachlor were low because of the masking effect of plant lipids present in the extracts. Burchfield and Storrs (12) and Burchfield and Hartzell (10) reported that allethrin, lindane, methoxychlor, Dilan, parathion, TDE, dieldrin, DDT, heptachlor, chlordane, Strobane, aldrin, and toxaphene will inhibit the photomigration of 50% of the mosquito larvae in an hour or less at concentrations of 1 p.p.m. and all of them, except TDE, Strobane and toxaphene, at 0.1 p.p.m. The optimum bioassay range was given for each toxicant tested. Not all physiologically active materials are effective in the determination of $T_{50}$, which is the time required to inactivate 50% of larvae. Nicotine and sodium arsenate will not inactivate larvae at 10 p.p.m. in less than 5 hours. Sodium cyanide is effective in 40 to 50 minutes at 10 p.p.m. However, aside from some of the acaricides, rotenone is the only important liposoluble insecticide so far tested which failed to inactivate larvae in the $T_{50}$ test. The evidence indicates that the test is more or less specific for liposoluble compounds which act as neural or muscular poisons.

## D. OTHER INSECTS

Fleming, Coles, and Maines (40) developed a tentative bioassay method for soil residues of chlordane and DDT, alone and in combination, using *Macrocentrus ancylivorus* Roh. or Japanese beetle grubs, *Popillia japonica* Newm., as test insects. At the rate of 10–20 pounds per acre of chlordane or DDT, the standard deviation of the analysis is about one pound per acre. Aldrin, dieldrin and other chlorinated insecticides in soil may also be determined biologically (40,43). By the same method, Fleming and Maines (41,42) determined DDT and chlordane in soils using Japanese beetle larvae. Lange and Carlson (66) used the Pacific Coast wireworm, *Limonius canus* Lec., to bioassay several insecticides in soil samples. In the search for more sensitive insects for bioassay of insecticide residues (101), several species of common insects were also tested. The $LD_{50}$'s for dieldrin were 1,2, >2, and 3 $\gamma$ per jar for young German cockroach, *Blattella germanica* (L.), confused flour beetle, *Tribolium confusum* Duv., red harvest ant, *Pogonomyrmex barbatus* (F. Smith), and young large milkweed bug, *Oncopeltus fasciatus* (Dall.), respectively. Ward and Burt (108) exposed *Tribolium castaneum* (Hbst.) directly to leaf discs for the bioassay of DDT on foliage.

## E. MICROCRUSTACEANS

Before the use of daphnids for bioassaying insecticide residues, *Daphnia magna* has been studied by Anderson (1,3) and other workers for testing the toxicity of various salts in relation to the study of industrial wastes. Anderson (1) pointed out many advantages of using *D. magna* as a test animal. Daphnids are small, reaching a maximum size of 5 mm., so that a great many can be reared in a small space. They have a life span of about two months when reared at 25°C. They are easy to culture, requiring only water containing bacteria or their equivalent for food. They give birth to young within their first week of life and give rise to new broods every two or three days throughout their lives. Since daphnids are most sensitive to toxic substances during their first instar the threshold concentrations for mortality can be established with greater certainty. Anderson (3) also reviewed the results of other investigators on several species of plankton crustacea.

Since *D. magna* is very sensitive to various salts, the threshold concentrations for their immobilization, as determined by Anderson (3), were 0.027, 0.039, 0.0051, 0.006, and 0.0026 p.p.m. for cupric chloride, cupric ammonium chloride, silver nitrate, mercuric chloride and cadmium chloride, respectively. The high sensitivity of daphnids to copper and mercury compounds suggests their use for the bioassay of certain fungicide residues. Anderson (2) also reported that 50% of daphnids were immobilized by 0.001 p.p.m. of DDT in 32 hours or less. The same species was also used by Wasserburger (109) as a test animal for the determination of traces of DDT, BHC and parathion. It is more sensitive than *Musca* or *Drosophila*. Kocher, Roth and Trebaux (64) used another species of daphnid, *Daphnia pulex* de Geer, for the bioassay of Diazinon on cauliflower, Isolan on peaches, and for chlordane, dieldrin, aldrin, BHC and DDT. For the detection of contact insecticides using daphnids, Pfaff (88) noticed that each of the six chlorinated hydrocarbons caused spasmodic movements in the early stages of poisoning, in contrast to circular movements observed with 16 organic phosphorus compounds. Close study of tests with standards permits various toxicants to be recognized by differences in their effects. As little as $10^{-8}$ g. of active substance can be determined.

Michael, Thompson and Abramovitz (76,77) found that brine shrimp, *Artemia salina*, a salt water crustacean about 12 mm. in length showed promise as a test organism for the detection of toxicants. Brine shrimp eggs are obtainable at most tropical fish stores and will remain viable for several years. This is a distinct advantage, since it eliminates much of the time in rearing test organisms. Two milliliters of an acetone suspension in a standard salt solution are placed in 10 by 75 mm. test tube, together with five three-week-old adult brine shrimps. The time when the shrimps are unable to swim indicates the amount of toxic material present. When 0.01 p.p.m. of toxicant was tested as a suspension in a standard salt solution, the time required for shrimps to lose swimming ability was 1,2,2,3,18,18, and 18 hours for methoxychlor, DDT, lindane, chlordane, toxaphene, endrin and Bayer 17147, respectively (76).

### F. FISH

Pagan and Hageman (84) showed that the lower limit of detection of DDT was about 0.025 p.p.m. using guppies, *Lebistes reticulatus* Peters, as a test animal. This concentration of DDT produced about 15% mortality in 24 hours.

### G. OTHER ORGANISMS

Attempts were made by Angelotti *et al.* (4) to find bacteria, yeast and protozoa sensitive to nabam, DDT, BHC, lindane or parathion for detecting fungicide and insecticide residues. In general, results were negative. However, *Micrococcus pyogenes* var. *epidermidis* showed a graded response to nabam.

## VI. Preparation of Extracts for Bioassay

The preparation of extracts for bioassay is not essentially different from that for chemical analysis. Generally, the extracts prepared for chemical analysis are equally applicable to bioassay (10,32,45, 103) and vice versa (80,90). Recently, Gunther and Blinn (48) reviewed and discussed the sampling, processing of samples, extraction, concentration and cleanup procedures for residue analysis. Since most of these studies were used for chemical analyses, they will not be reviewed here unless they are related to bioassay.

It is not an easy and simple procedure to obtain representative field-treated samples for residue analyses. In field experiments the deposits vary greatly. It is necessary to secure a large sample which represents the bulk of the treated material. The sampling method for the residue analysis of one product does not necessarily apply to the residues of other products. Each residue study has its own sampling problem. Therefore, in order to obtain accurate results, a proper sampling method should be developed for each material. Fahey (33) reviewed the literature from 1925 to 1952 relating to sampling of crops for spray residue analysis. Fleming, Coles and Maines (40) reported a method for sampling and preparing soil for bioassay. Patterson and Lehman (86) discussed procedures for sampling, extraction and assay of pesticide residues which are applicable to both bioassay and chemical analysis.

Cross contamination of toxicants by drift is a very serious problem in small plot experiments. Due to the non-specific nature of bioassay methods, contamination during or after treatment is more serious than that for specific chemical analysis. Sun and Sun (103) reported that one "untreated" alfalfa sample submitted for bioassay contained as much as several parts per million of a toxicant when it was collected immediately after treatment of adjacent plots. In order to avoid field contamination and to obtain more reliable results, untreated checks should be taken before treatment or they should be protected from the drift. Besides, large field plots are preferable to small ones; sprays are preferable to dusts; and calm weather is preferable to windy weather during treatment.

Successful recovery of toxicants from representative field samples often depends upon immediate and quantitative stripping of residues. This consists of subsampling, grinding or macerating and the use of proper solvents and extraction processes. A delay in extraction would be likely to reduce the true residues of the more volatile or unstable compounds (55). Although a good part of pesticide deposits on plants is readily lost through volatilization and weathering, a trace of toxicant will persist on or in plants as absorbed or solubilized residues in plant waxes (6,57). The insecticide may also penetrate or be translocated in the plants (16,75). Contrary to the early belief of some workers that microquantities of pesticides can be easily extracted from plant or animal tissues with relatively large quantities of solvents, toxicants are often not quantitatively recovered. Therefore, in recovery studies, the toxicant should be added before extraction, and not after or during extraction. Because of the differences in the properties of toxicants and the nature of samples, a satisfactory extraction method for one toxicant in one material can not be applied, without proof, to another toxicant in the same or different materials. For example, aldrin can be extracted with hexane from different types of soil. However, dieldrin can not be quantitatively removed by the same extraction method from rich, humus soil without the addition of polar solvents, such as isopropyl alcohol and acetone. Compounds, such as heptachlor and aldrin, which are readily metabolized (5,26,27) should be extracted and chromatographed very carefully without losing the toxic metabolites which have quite different physical and chemical properties.

Hoskins and Messenger (58) warned that there is always the possibility that a chemical present in some part of the body after ingestion will be harder to extract than when freshly mixed with the tissue. Hence, precautions such as fine subdivision, long extraction, and use of a good solvent should always be taken. Realizing the impossibility of duplicating the natural conditions of insecticide residues, Terriere and Ingalsbe (104) tried to approximate these conditions by adding an acetone solution of DDT or dieldrin to homogenized potatoes; they were allowed to stand for five hours before proceeding with the extraction.

Feeding methods (38,85,103) can detect pesticide residues without extraction. However, difficulties did occur in the bioassay of DDT (84), toxaphene and chlordane (14) in milk without extraction. In most cases, extraction is an unavoidable procedure for residue analysis.

Selection of proper solvents is of primary importance in the success of an extraction method. To reduce the total extractives, hydrocarbon solvents, such as hexane, petroleum ether, etc., are often used. Better solvents, such as benzene and acetone, would give a more complete extraction of many residues, yet they may dissolve too much extractives which would seriously interfere with bioassay (32,40, 58,59,104). Samples with high water content should be extracted with water-miscible solvents, such as isopropyl alcohol or with mixtures containing such solvents.

In the process of extraction, large quantities of extractives are usually dissolved in the solvents. The extractives will dilute the active toxicant in the deposit prepared for bioassay and may mask the toxicity of insecticide residues (10,11,14,40,52,58,59,60,68,103). Natural toxicants may increase the toxicity (59,60,67,68,79,103,104). Therefore, proper cleanup is necessary for extracts of many materials in order to obtain accurate results and high sensitivity.

Sulfonation of the unsaturated fats, waxes and colored materials by treatment with sulfuric acid can be used only for acid-stable toxicants, such as DDT (14,45,58). Saponification of oils and fats can be applied only to alkali-stable toxicants, such as aldrin, dieldrin, isodrin and endrin (63,93). Because they are less drastic, absorption columns (11,32,58,59,77) and solvent partition techniques (12,59,63) have more general use than acid treatment or saponification in the cleanup of extracts.

Laug (67,68) ground wet or dehydrated animal tissues with a small quantity of washed sea sand, extracted the mixture with successive 10 ml. portions of ether and then decanted the extract without filtration. A portion of the ether extract was used for bioassay without cleanup. The extracts of liver, kidney and brain were particularly toxic to house flies and gave unreliable results.

Bushland (14) apparently obtained good recovery when three butterfat samples containing 25 p.p.m. of lindane were dissolved in chloroform and treated with a sulfuric acid-fuming sulfuric acid mixture. However, with the same method he failed to kill any larvae at the highest test concentration of DDT and TDE, although all the standards made from acetone solution caused complete mortality at that and at lower concentrations. Apparently the acid treatment did not completely remove the extractives, the amount of which was not measured or compensated for in the standard.

Furman and Hoskins (45) and Hoskins and Messenger (58) reported that treatment of a chloroform extract containing benzene hexachloride or DDT with concentrated sulfuric acid reduced the interference due to plant or animal extractives. Hoskins and Messenger (58) also found that interfering extractives from some plants can be removed without loss of parathion or DDT by adding a chloroform extract to a short column containing two parts of Attaclay plus one part of Hyflo Super-Cel, followed by rinsing with an equal volume of pure chloroform.

Hoskins (57) extracted benzene hexachloride with benzene from plant and animal tissues by rolling and shaking the mixture for five minutes. The residue so determined may be called the surface residue as distinguished from that removed from within the sample. Total residues were recovered from processed vegetables and animal products by intimately mixing the finely subdivided sample with solvent in a Waring blendor. The completeness of extraction of residues was studied in several cases. The general result was that the additional recovery in the second portion of solvent was only a very few per cent of that in the first portion. This was confirmed by tests for recovery of added insecticides.

Hoskins, Schiller and Erwin (59) and Erwin, Schiller, and Hoskins

(32) emphasized that insecticide residues in samples with high water content cannot be effectively extracted from the tissues with water-insoluble solvents, even though such solvents may be satisfactory for recovering added toxicant. The difference is that the added toxicant is dispersed among the tissues rather than actually in them. A 1:1 mixture of solvent-alcohol did extract up to five times as much insecticide residues as with benzene alone. In order to remove lipids from plant extracts Burchfield and Storrs (12) used the technique of partition of insecticides between *N,N*-dimethylformamide and hexane. Some insecticides accumulate in the more polar (acetonitrile) phase, others in the amide. The lipids remain in the hexane.

Using a technique similar to that of Jones and Riddick (61), Hoskins *et al.* (59) extracted a petroleum ether solution with three volumes of acetonitrile. The acetonitrile solution was diluted with three volumes of water and the resulting mixture was extracted with petroleum ether. Ninety per cent or higher recovery was obtained with heptachlor, parathion, and EPN added at the rate of 0.1 p.p.m. Recovery of methoxychlor, DDT, Dilan and rotenone was satisfactory but that for aldrin, toxaphene and chlordane was erratic. While the acetonitrile extraction eliminated many extractives and was a satisfactory method for purification for bean or carrot, it failed badly for canned spinach or sauerkraut. Petroleum ether and acetonitrile were also used by Klein *et al.* (63) to extract endrin from leafy vegetables. Relatively small amounts of waxes and other plant materials were extracted by acetonitrile.

In order to remove extractives, Hoskins *et al.* (59) and Erwin *et al.* (32) used a reverse-phase partition column which was prepared by coating chromatographic alumina with a 2 to 1 mixture of petrolatum and low melting paraffin wax. With an extract from 50–60 g. of canned spinach (59) the following toxicants were recovered at 90–100% with 60% acetonitrile as an eluent of the "wax column": DDT, TDE, methoxychlor, BHC, lindane, chlordane, heptachlor, toxaphene, parathion, malathion, and Diazinon; at 70–90%, dieldrin and endrin; and at about 65%, Dilan. Aldrin and isodrin are unique in that they require 70–75% acetonitrile but then give high recovery. This column method has been successfully used for residue analysis of many plant and animal products, but not for oils. Michael *et al.* (77) also used this method for preparing extracts for bioassay using brine shrimps.

Hoskins *et al.* (59) also made preliminary tests with "plastic columns" prepared by coating alumina with polyethylene, polystyrene or high polymer grade Bakelite DTNH. No special advantages were obtained over the "wax column."

Carter, Nelson and Gersdorff (18) extracted toxaphene residues from hay with benzene. After the removal of benzene, the residues were taken up in deodorized kerosene and tested against house flies by the turntable method.

Benzene was also used by Nolan and Wilcoxon (79) to extract parathion residues from plant materials, and by Hartzell and Storrs (52) to extract heptachlor, aldrin, dieldrin, chlordane, lindane, methoxychlor, TDE, and toxaphene from processed foods. In general, the insecticide toxicity was reduced by the presence of plant material. At higher concentrations these extractives appear to be toxic to mosquito larvae. Apparently, a proper cleanup method is necessary to remove these interfering materials. Starnes (97) claimed that lindane and parathion residues obtained by the aqueous extraction method were not significantly different from those obtained from benzene extracts described by Nolan and Wilcoxon (79).

Fleming *et al.* (40) extracted soil samples with a mixture of benzene-isopropanol (2:1) for 30 minutes on a shaking machine. The extract was filtered and used for bioassay. Since extractives were found to interfere with the bioassay results, activated charcoal was used to remove them. However, charcoal also removed two-thirds of the chlordane and half of the DDT, regardless of the concentration of the toxicants. Other solvents, including methanol, ethanol, isopropanol, acetone and petroleum ether, were used for extraction, but did not improve the situation. The inhibiting constituents could not be removed by subjecting the extracts to a temperature of −17°C. nor by washing with dilute sulfuric acid or with aqueous solutions of sodium carbonate.

In the study of translocation of soil insecticides, Terriere and Ingalsbe (104) homogenized raw potato in water and added benzene at the rate of 1 ml. per gram of tissue. The mixture was shaken for one hour and the resulting emulsion was broken with anhydrous sodium sulfate. The natural toxicant in the benzene extract was removed by treating with 0.2% sodium hydroxide without apparent change to any insecticide residue present.

In the photomigration method Hartzell *et al.* (53) reported that the extractions of heptachlor and chlordane from crops were made with *n*-pentane; plant pigments were removed by chromatographic absorption on Florisil. For chlordane the concentrated solutions were passed through alumina and the lipids passed through the column. The absorbed chlordane was then eluted with ethyl ether.

In the preparation of extracts for bioassay with mosquito larvae, the material is usually macerated in a Waring blendor and extracted with benzene (52,79), carbon tetrachloride (78), *n*-hexane (11) or *n*-pentane (10,53). The extract is first purified and then carefully evaporated to dryness. The residue is redissolved in acetone and dispersed in water for bioassay.

Hartzell *et al.* (53) mentioned that steam distillation is particularly attractive for isolating the more volatile insecticides, such as lindane and heptachlor. The steam distillate can be collected and used for bioassay without further extraction or concentration. By carrying out the distillation in a current of steam, recoveries of heptachlor and lindane were 100%, while the yield of parathion, chlordane, aldrin and methoxychlor were less satisfactory.

Hexane has been extensively used for the extraction of aldrin, dieldrin, isodrin and endrin from various materials for bioassay (103) using the following procedures (93). The sample is subdivided to fine particles and extracted with hexane or hexane-isopropyl alcohol (3:1) in a tumbling machine. Some extracts can be used for bioassay without cleanup, while others have to be chromatographed in order to obtain a required sensitivity. For oils, fats and materials with high glyceride contents, the samples or extracts are saponified with an alcoholic potassium hydroxide solution. The extract is then washed, concentrated and used for bioassay. These procedures have been used for 18 types of fruits, 26 kinds of vegetables, 20 other crops, 13 varieties of animal tissues and 4 other materials. Occasional modifications are necessary when problems arise from those extracts containing large amounts of waxes. Both purified pentane and hexane extracts were used by Tressler and Jenkins (107) for bioassaying 6 insecticide residues using house flies.

## VII. Calculation and Interpretation of Bioassay Results

Methods of calculating bioassay results are not as simple and straightforward as those for chemical analysis. Same data are subject to different interpretation. The difference in the interpretation may be largely due to biological variations as well as due to the methods of calculation. Sun and Sun (103) mentioned four methods for the calculation of various amounts of insecticide residues. The application of each method and the interpretation of results were discussed on the basis of the amounts of residues and the design of bioassay experiments.

The concentration of a toxicant used in the bioassay is often expressed as parts per million for aqueous suspensions (52,79) and macerated food (38,85,101) and as micrograms per test container (58,103) or micrograms per gram of insects (22,67,68) for dry-film methods. The response of the organisms may be expressed as mortality, time of response, or other physiological reaction. For routine analysis, corrections for check mortality are not necessary, because they do not give significant differences in the final calculation (100). The standard dosage-response (or dosage-mortality) data are plotted as log-probits (8) to produce a straight line by visual inspection. However, statistical computations of the probit regression line (36) should be used for more accurate calculation. Results for unknown amounts of a toxicant are read graphically by reference to the standard curve which must be constructed for each series of analysis (11,22,40,44,53, 63,67,77,85,96,98,103). Due to the variability of the slope of the dosage-mortality line, this method of calculation gives more variations at low and high mortalities. Therefore, only a certain range of mortalities should be used (10,32,63,96,98,101,103).

A rapid analysis by the ranking method (110) has often been used for determining the significance of difference (85,101,103) for low residues.

In addition to the above two methods of calculation, Sun and Sun (103) used an "$LD_{50}$ method," for the determination of traces of residues. If an extract or sample does not contain enough toxicant to give a moderate kill of test organisms, the same amounts of the same toxicant are added to both the standard and sample (98,103). The difference in $LD_{50}$'s of the standard and the treated sample represents the quantity of toxicants in the volume of treated sample. The result is positive when the $LD_{50}$ of the standard is greater than that of the sample. This method of detecting minute, sub-lethal quantities of residues can be applied to other bioassay methods.

Based on the negative phototaxic response of mosquito larvae, Burchfield, Hilchey and Storrs (11) devised an equation to calculate the time required to inactive 50% of the larvae.

$$T_{50} = \sqrt{\tau^2 + A/(c - c_0)^n}$$

where $n$ and $A$ are constants, $\tau$ is the minimum inactivation time, $c_0$ the critical concentration, and $c$ the initial concentration of the insecticide suspension. The relationship between the $T_{50}$ and the concentration of insecticide is not linear (10), but it is usually satisfactory to interpolate results from a graph of the logarithm of the $T_{50}$ against the reciprocal of the concentration in parts per million (10,53).

Michael *et al.* (77) reported that time-flotation graphs can be prepared from data obtained by exposing brine shrimps to known amounts of pure toxicants or of toxicants recovered from the same kind of sample. By comparison with data obtained from the test samples the amount of toxicant present can be read directly.

After obtaining the amount of residue in each test, the standard method of calculation can be used to express the results in terms of parts per million or other units. By direct-feeding methods, the data obtained are often expressed as parts per million of toxicants without further calculation.

To determine the sensitivity of a given experiment, required to produce a given sensitivity, the following equation was devised by Sun and Sun (103).

$$\text{Sensitivity} = \pm \frac{D \times F}{R \times V}$$

where $D$ is the $LD_{50}$ of a standard curve expressed as micrograms of a toxicant per jar; $F$ is a factor of accuracy which may vary from 0.2 to 0.1, depending upon the number of replicates and the accuracy of results; $R$ is the extraction ratio expressed as the number of grams of a sample per milliliter of the final extract; and V is the volume of a treated extract in milliliters added into each jar. The same equation can also be used to calculate the approximate size of a sample and the volume of extract in each jar to produce a desired sensitivity. A modified equation was used for calculating the sensitivity of feeding methods (98).

## VIII. Factors Affecting Bioassay

The success of bioassay of pesticide residues depends largely upon the knowledge of various factors affecting results, and on the development of means to correct or to control them. Many factors affecting bioassay have been reported and discussed by various workers (11, 38,60,67,102). Some factors may affect one bioassay method more than another, or may affect one toxicant, or one test organism more than another.

### A. TISSUE EXTRACTIVES

Tissue extractives may mask the toxicity of residues by simple dilution or by absorption, and may increase the toxicity if they contain natural toxic materials. The toxicity to house flies of extractives from liver, kidney and brain was reported by Laug (67,68). Using the same test insect, no significant toxicity was observed in a two-day exposure by Sun, Pankaskie, and Sun (102) on extracts obtained from beef fat, pork liver, corn oil, lard or beeswax. However, 0.2 ml. of orange oil killed 98% of flies in one day. Variations in the amounts of orange oil and in the time elapsed before the addition of flies resulted in unusual variations in fly mortalities when aldrin and dieldrin were tested in the presence of the oil. The extract of unripe peaches at certain dilutions was found by Hartzell (51) to be toxic to mosquito larvae. Potato extract was reported by Nolan and Wilcoxon (79) and Terriere and Ingalsbe (104) to be toxic to mosquito larvae. But this was not experienced in the dry-film method using house flies (103).

The masking effect of tissue extractives is not as serious as the toxicity of some extractives. However, even when the masking effect of extractives can be compensated for by including equal amounts in the reference standards, the sensitivity of bioassay is greatly reduced (10,53,58,103). Hoskins and Messenger (58) reported that the $LD_{50}$ for house flies is 14 $\gamma$ for parathion in the presence of cabbage extractives as compared to 0.56 $\gamma$ without extractives. Sun and Sun (103) found that 20 mg. of corn oil, lard, and wax in each jar decreased the toxicity of dieldrin to house flies to about two-thirds, one-third, and one-fourth, respectively; 2.5 and 20 mg. of wax in a jar can reduce the fly mortality caused by 3 $\gamma$ of dieldrin from 84% (no wax) to 41% and 17%, respectively. In the presence of 100 p.p.m. olive oil, Hartzell *et al.* (53) estimated a loss of 90% toxicity of 0.1 p.p.m. heptachlor solution. Certain plant extractives do not mask the toxicity as much as the others. Sun, Pankaskie and Sun (102) reported that the $LD_{50}$ values were 4.3 and 5.0 $\gamma$, respectively, for aldrin in alfalfa and wheat extracts containing 4 and 50 mg. of extractives per jar. Hartzell *et al.* (53) observed that cholesterol, cetyl alcohol, stearic acid, and crude hexane extracts of green beans had no noticeable effect at 1 p.p.m. and repressed the rate of insecticidal action only moderately at 10 p.p.m.

### B. TEST ORGANISMS

The responses of test organisms to toxicants vary with species, stage, age, sex and size. The susceptibility of the test organism and the consistency of its response affect the sensitivity and accuracy of bioassay results. The sensitivities of several species of insects and other organisms to various toxicants are listed in Table II. Mosquito larvae (11,51,52) and pomace flies (38,85,101) are more sensitive than other insects.

Laug (67) reported that male house flies are more susceptible than female house flies and more DDT is required to kill a given weight of large flies than an equal weight of small flies. The $LD_{50}$ varies from 1.3 to 3.8 mg. per kg. of flies. Similar differences were reported by Hoskins and Messenger (58) (Table II). By over-seeding eggs in the larval medium, Sun, Pankaskie, and Sun (102) were able to breed small house flies about four-tenths of the weight of flies reared in uncrowded media. When exposed to 1 $\gamma$ of dieldrin, 29 and 14% of normal male and female flies died; but only 0.5 $\gamma$ was required to kill 73 and 51% of the small male and female flies. Sun and Sun (98) revealed that young house flies are more susceptible than older ones. At a dosage level of 3 $\gamma$ of dieldrin per jar, 92, 82, 56 and 38 per cent of 1, 2, 3, and 4-day old flies, respectively, were killed (102).

Results obtained by Fisher and Smallman (38) indicate that there are no appreciable differences in the $LD_{50}$'s of aldrin against 1 to 4-day old *Drosophila melanogaster*. However, males are more susceptible than females.

Burchfield *et al.* (11), and Burchfield and Hartzell (10) emphasized that, in order to obtain reproducible results, mosquito larvae used for photomigration tests should be reared under carefully standardized conditions. Second instar larvae at an age of 20 to 22 hours are generally employed. Newman (78) was able to reduce the $LD_{50}$

for parathion from 0.5 $\gamma$ to 0.002 $\gamma$ per tube by using the first instar larvae in place of the third instar and by reducing the volume of the test solution from 50 to 1.5 ml. Terriere and Ingalsbe (104) pointed out that the use of total lack of movement upon stimulation as a criterion of death reduces the sensitivity. When a 15 ml. assay volume is used in place of 50 ml., the sensitivity is increased.

Fleming *et al.* (40) found that mixed sexes of *Macrocentrus ancylivorus* adults could be used for making biological assays but somewhat more consistent results were obtained when only one sex was used. The age of these adults up to 2 days old was not a factor affecting bioassay.

In toxicity experiments with *Daphnia* and other arthopods, Anderson (3) emphasized that exposure periods should be long enough so that all the experimental animals will have an opportunity to reach the molting stage. The importance of the molting period is substantiated by the marked inflections in the concentration-immobilization time curves for many compounds at the time of molting. Explanations for such difference in results are the increase in permeability of soft body integuments, the increase in contact area after molting, the inhibition of the molting process, and the development of abnormal integument resulting in death. These factors may also apply to mosquito larvae.

## C. TEST CONDITIONS

Laug (67) called attention to several physical factors which affect bioassay tests. The uptake of DDT by house flies increased with the time of contact; after a 4-hour exposure flies removed all of the DDT deposit. Shorter exposure periods gave higher $LD_{50}$'s. The addition of small quantities of moisture to a DDT deposit shortened the toxic induction period and produced a measurable decrease in the $LD_{50}$. Natural light as compared to darkness made the flies more sensitive to DDT. Lowered temperatures made the flies more sensitive to DDT. This is probably due to the negative temperature coefficient of this insecticide. Other toxicants having a positive temperature coefficient may be expected to show a reverse effect.

In the bioassay with house flies Hoskins, Witt and Erwin (60) reported that insecticides may be deposited in widely different forms with varying toxicities. For example, fresh DDT deposits will persist as small liquid droplets on the surface, but when a fly walks through them the droplets change to minute crystals. Liquid insecticides, such as parathion, are readily contacted and do not give trouble in house fly tests. They also discussed many factors affecting the contact between insecticide and test insects when persistent deposits are used in the small vial technique. These factors are the solid or liquid nature of the deposit, its uniformity over the exposed surface, the length of exposure, the portion of the insect's body reached, and the opportunity for loss of the adhering insecticides.

Experimenting with lindane deposits against house flies, Hoskins *et al.* (59) concluded that no two vials had identical size or distribution of lindane crystals. Flies picked up different amounts of crystals and their mortality varied in an unpredictable manner. A small amount of light spray oil prevents the formation of crystals and gives a uniform distribution of insecticide over the entire surface.

Burchfield, Hilchey and Storrs (11) studied the test conditions affecting the photomigration of mosquito larvae. A volume of 100 ml. gives a proper depth of water for larval migration. A migration time of one minute using a 500-watt light and a distance of three inches give satisfactory results. Since the principle of this method depends upon the negative phototaxic response of larvae, the exposure of larval culture to lights of different kinds and intensities has an important bearing on the accuracy of results. From the standpoints of accuracy and convenience, unknown solutions are best run within a 25 to 100-minute range. When the time is short, errors in estimating concentration will be large, whereas when the time is long, the natural mortality of the larvae and the presence of foreign toxicants may tend to give misleading results.

### D. CONTAMINATION

Accidental contamination with toxicants, other than dust or spray residues, is the most serious problem in bioassay. Many workers have been very much discouraged and confused by contamination which can be introduced in the field, in the handling and processing of materials, in extraction, and in the process of bioassay. In the laboratory, contamination of equipment is a common and serious problem. For mosquito bioassay Hartzell and Storrs (52) and Burchfield *et al.* (11) treated equipment with hot trisodium phosphate solution followed with sulfuric acid-dichromate cleaning solution. Sun and Sun (103) washed jars thoroughly with a household cleanser, rinsed them with water, and heated them overnight at 90°C. or higher in order to eliminate traces of toxicant. To avoid such contamination, it is absolutely essential to have separate equipment, and even an individual room isolated from other pesticide testing work.

Considering all the above factors that affect the response of organisms to the toxicant, the task of standardizing all conditions and variables appears unsurmountable. Therefore, it was generally agreed that the standard should be included in each series of bioassay tests. Factors which affect the response of organisms in the unknowns should also affect those in the standard to the same degree. Such a similar response to various factors was illustrated by the consistency of toxicity index (100) when an insecticide and the standard were sprayed with the following variables: volume of spray, wind velocity in the spray tunnel, fineness of spray and spraying pressure, criterion of death, length of time between spraying and counting mortality, age of flies, size of flies, and mortality of flies in the control. In the bioassay of insecticide residues, changes of the following factors did not appreciably affect the final results (94,98): age of flies, size of flies, exposure time, criterion of death, amount of extractives, methods of making deposits, variation of room temperature, etc. For example, when the house fly mortality was counted at 28 and 46 hours of exposure, the toxic residue of the same sample was found to be 2.6 and 2.8 p.p.m., respectively. These relative, proportional changes by testing one series of samples and the standard at the same time with the same culture without strict standardization give bioassay a distinct advantage.

Sensitivity of bioassay methods varies greatly with the toxicity of pesticides to test organisms. With less sensitive organisms, it is often difficult to obtain the desired sensitivity. However, sensitivity can be improved by an increase in sample weight used for analysis, by the removal of extractives from the extract, by the increase in the volume of extract used for each test, by the increase of contact time between insects and toxic deposits, by using longer intervals of observing mortality, by using smaller, younger insects and/or males (98,103). Better sensitivity can also be obtained by adding a small amount of the same toxicant to a test sample (10,98,103). A proper selection of the above factors can overcome the difficulties of obtaining a required sensitivity when a standard bioassay method is used.

## IX. Bioassay of Mixed Residues

Analysis of pesticide residues with an unknown history of treatment is the most difficult problem. Although Fahey (34) devised a method of calculating mixed insecticide residues on the basis of the sum of the per cent tolerance for each chemical, this method is applicable only when the residue for each toxicant is determined. The insecticide residues on crops reaching the market may consist of mixtures of unknown composition. It would be difficult to select the proper chemical method to determine the residues; it is also practically impossible to make a comprehensive analysis of all insecticides (10,28,103). A preliminary bioassay will indicate the presence or absence of a wide variety of toxicants. In many cases only the bioassay result is needed when the total toxicity is low.

Although bioassay is generally non-specific in nature, certain pesticide residues may be separated and determined by bioassay methods. The principles which have been or could be applied to the separation and bioassay of mixed residues may be physical, chemical or biological in nature.

Laug (68) claimed that the existing chemical methods used at that time did not differentiate between mixtures of the isomers of benzene hexachloride. However, he found that it required approximately 100 to 200 times of the *alpha, beta, delta* and *epsilon* isomers of benzene hexachloride to effect the same kill of house flies as with the *gamma* isomer. Therefore, he concluded that the toxic response of the house fly to the pure *gamma* isomer was characteristic. It may be assumed that the toxicant in the tissues of rats ingesting *gamma* isomer was the *gamma* isomer.

Based on the difference in volatility and the speed of action, Fleming, Coles, and Maines (40) bioassayed a mixture of chlordane and DDT in soil extracts. Chlordane was first assayed and then the filter paper was successively dried and wetted with water, usually 10 or 12 times, before assaying DDT. The difference in the total organic chloride as determined biologically or chemically was less than one pound per acre with an average variation of about 10%. They concluded that the bioassay method had detected DDT and chlordane in about the same proportion as they occurred in the soil. The same principle may be applicable to the analysis of other mixtures containing one volatile and one non-volatile toxicant with different speeds of action to certain test organism. However, the technique would be tricky and needs skillful handling.

In order to detect several insecticide residues in one sample at a minimum cost in time and money, Davidow and Sabatino (28) determined the characteristic time pattern of conversions of goldfish to 9 chlorinated insecticides. Positive results were obtained with food containing 7 p.p.m. DDT, 5 p.p.m. lindane, 7 p.p.m. toxaphene, 14 p.p.m. methoxychlor, and 7 p.p.m. Dilan. However, the fish failed to respond positively to 0.1 p.p.m. of heptachlor, chlordane, aldrin or dieldrin. They suggested that goldfish may be used for the rapid sorting of foods contaminated with excess amounts of DDT, lindane, toxaphene, methoxychlor and Dilan.

Burchfield and Storrs (13) noticed that *Aedes aegypti* larvae treated with DDT contract spasmodically on exposure to the migration light but appear unable to move away from it. When treated with parathion they react sluggishly and move away from the source at a reduced rate, but in advanced stages may even move toward it. With experience it may be possible to guess the nature of an insecticide by the response of the larvae during photomigration.

A proper chromatographic column not only can remove a great deal of extractives, but can also separate compounds which show different degrees of polarity. Erwin, Schiller and Hoskins (32) listed three groups of insecticides which can be carried through the granulated paraffin-cellulose-starch or the paraffin-vaseline-alumina column with three different concentrations of acetonitrile. Rotenone, malathion, Dilan, parathion, lindane, methoxychlor and part of dieldrin will be eluted by a 40% acetonitrile solution; dieldrin (part), DDT, chlordane, heptachlor, endrin, and toxaphene, by 60% acetonitrile; and aldrin and isodrin, by 75% acetonitrile. This separation aids greatly in determining what is present. The separation is fairly sharp when no extractives are in the solution and is less distinct as these increase in amount. If a mixed residue contains toxicants in different groups, it is possible to bioassay each separately.

Mixed residues of aldrin and dieldrin, or isodrin and endrin in insect, animal or plant tissues have also been successfully separated, by passing the hexane extract through an Attasol or Attaclay column. Aldrin passes through the column, but dieldrin will be retained and can be washed off with polar solvents. The separation is quantitative and has been used for bioassaying these mixtures (94).

Differences in the stability of pesticides to chemicals may be used for destroying the toxicity of one or more undesirable residues, leaving the only stable toxicant for bioassay. To prove the presence of endrin residues in leafy vegetables, Klein *et al.* (63) reported that endrin, dieldrin, aldrin and presumably isodrin may be refluxed with 2% potassium hydroxide without change of insecticidal activity. The same treatment causes DDT, BHC, methoxychlor, parathion, and malathion to degrade into compounds which are comparatively inactive. Chlordane, heptachlor, and toxaphene, however, are only partly degraded to insecticidally inactive compounds. Endrin is decomposed by strong acid treatment, but DDT is rather stable. By a proper selection and application of the methods relative to the acid and alkali stability of pesticides, one toxicant may be bioassayed without the interference of the other.

In the assay of aldrin or dieldrin residues on crops, DDT or parathion has been occasionally used at the same time for controlling a combination of insect pests. Before the development of a sensitive chemical method for analyzing aldrin and dieldrin, the mixed residues were prepared by bioassay by refluxing with a 6.5% alcoholic potassium hydroxide solution at 75–80°C. for two hours. Reasonable quantities of DDT, lindane, and parathion did not interfere with the bioassay of aldrin or dieldrin after alkaline treatment. However, only 86% of chlordane toxicity and 67% of toxaphene toxicity were destroyed. By increasing the alkaline concentration to 12%, chlordane lost more than 90% of its toxicity, but there was no further significant loss of the toxicity of toxaphene (94).

On the basis of known biological responses, other devices may also be used to determine qualitatively or quantitatively a mixture of certain known pesticides. Most phosphorus insecticides act much faster than chlorinated ones on house flies and other insects. The difference in the speed of action may separate these two types of residues. A strain of house flies resistant to chlorinated insecticides may be used for the bioassay of phosphorus insecticides in the presence of chlorinated insecticides. Cholinesterase which is inhibited by many phosphorus compounds, but not by chlorinated hydrocarbons, could be used for assaying phosphorus toxicants in the presence of chlorinated ones. Using a combination of the physical, chemical and biological means of separation and detection, a bioassay method can be used more specifically for estimating toxicants in a mixture. After all, the specificity of many chemical methods depends largely upon the separation of interfering materials which may be extractives or pesticides.

## X. Bioassay and Metabolic Products

Recent studies on insecticides have revealed that many toxicants are metabolically changed in animals and plants. Detoxification has been recognized as a common natural defense to toxic foreign materials; however, some pesticides have been metabolized to more toxic materials. The end products may retain the reactive group which will still give the original analytical chemical reaction, but they may be less or non-toxic. On the other hand, a compound may be altered to a more toxic metabolite which will not react with the reagents used for detecting the parent compound. Thus, metabolic changes complicate the analysis of pesticides. Under such conditions bioassay methods could give a measure of total toxicity and an estimate of the approximate degree of contamination can be made. But, bioassay will not give any specific information as to the composition of the toxic residue. A proper combination of two or more methods for analyzing the same sample should give enough information to discover whether possible changes are taking place. The following illustrate the metabolic changes of several insecticides, the study of which is more or less related to bioassay.

Parathion is known to be oxidized *in vivo* to the oxygen analogue, para-oxon, which is a potent inhibitor of cholinesterase. Metcalf and March (74) reported that the $LD_{50}$'s of parathion and para-oxon are 3.5 and 0.6 $\gamma$ per gram of worker bees, while the $IN_{50}$ values are $1 \times 10^{-6}$ and $1.9 \times 10^{-8}$ moles respectively for bee brain cholinesterase. The corresponding differences for house flies are smaller. Such metabolic change of parathion would give large differences in residue results when assayed with insects and with cholinesterase. Schradan and demeton are also reported to undergo metabolic changes.

The analysis of organically bound chlorine has been widely used for the determination of chlorinated insecticides. This method is as much lacking in specificity as bioassay methods. In some cases the chlorine content may be derived from breakdown products of the insecticides instead of the insecticides themselves (40,52). Therefore, one analytical method would be inadequate to identify a toxicant that is readily metabolized in tissues (Table III).

TABLE III

Comparison of Bioassay and Other Analyses of Insecticides with Possible Metabolic Changes

| Material | Treatment | Toxicants found, p.p.m. | | Reference |
|---|---|---|---|---|
| | | Bioassay[a] | Colorimetric analysis for original toxicant | |
| Rat fat | 25 p.p.m. aldrin in diet for 6–7 weeks | 46.8 | 2.5 | (5) |
| Sheep fat | Injection with aldrin, at 50 mg./kg. body weight | 55 | 7.5; $42.5^b$ | (5) |
| Beef fat | Injection with aldrin, at 50 mg./kg. body weight | 110 | 18.5; $93^c$ | (5) |
| Pig fat | Fed on aldrin-treated pasture (14.8 lb./acre) for 77 days | 2.7 | <0.1; $1.82^b$; $2.0^c$ | (5) |
| Milk | 66 mg. aldrin per day for 2 weeks | 1.7 | <0.1 | (5) |
| Butterfat | Aldrin-treated | 41 | <0.1; $49.5^b$; $38.6^c$ | (5) |
| Eggs | Hens fed with 88 p.p.m. aldrin in diet for 104 days | 48.7 | <0.1; $46.0^b$; $40.3^d$ | (5) |
| Carrots | 12 lb. aldrin per acre | 0.298 | 0.187; $0.134^b$; $0.296^c$ | (46) |
| Resistant house fly (surviving) | 15 $\gamma$ DDT per fly, by topical application | 0.50 $\gamma$ | 1.35 $\gamma^e$ | (72) |
| Resistant house fly (dead) | 15 $\gamma$ DDT per fly, by topical application | 0.50 $\gamma$ | 1.71 $\gamma^e$ | (72) |

[a] Based on total toxicity; calculated as the original toxicant.

[b] Colorimetric analysis for dieldrin.

[c] Total chlorine analysis; calculated as the original toxicant.

[d] Infrared analysis for dieldrin.

[e] Radioassay.

In the study of DDT in rabbit and rat tissues, Laug (67) observed that in the case of DDT in perirenal fat, the colorimetric and bioassay methods showed essential agreement. However, in the case of deterioration samples, the colorimetric method tended to give somewhat higher results. This is probably due to low toxicity of the DDT metabolites which may give a color reaction.

Lindquist *et al.* (72) found that, based on the radioactivity recovered and the toxicity of extracts to mosquito larvae, 50–81% of DDT was metabolized by DDT-resistant house flies. To confirm this, DDE and DDA, the known metabolites of DDT, were tested at 0.01 p.p.m. and were found to be non-toxic to mosquito larvae. They believed that the material that was toxic to the larvae was unmetabolized DDT.

From bioassay, radiometric assay, and colorimetric analyses, Robbins and Dahm (92) concluded that the fecal metabolites from American cockroaches were different from either DDT or DDE and did not possess the same physiological properties as DDT.

In the study of the storage of toxaphene in the fat of sheep and cattle, Diephuis and Dunn (29) mentioned that infrared and bioassay results agreed with the determination of organic chlorine. They concluded that toxaphene stored in the fat was unchanged; no comparable data were given, however.

Davidow and Radomski (26) and Radomski and Davidow (90) reported that heptachlor fed to dogs and rats is metabolically oxidized and stored as the epoxide in the fat. The epoxide metabolite has also been found in the milk of a dairy cow fed on heptachlor (27). Ely *et al.* (31) reported that from 0.0 to 5.7 p.p.m. of heptachlor epoxide was found in the cow milk as the daily feed of heptachlor increased from 0.30 to 3.78 mg./kg. Comparative intravenous toxicity measurements show that heptachlor epoxide is 2 to 4 times more toxic than heptachlor (86). In the colorimetric analysis Radomski and Davidow (90) revealed that there is no mutual interference between the two compounds because of the difference in absorption peaks. Heptachlor epoxide will be held in the column, but not heptachlor. If one is not aware of the change of heptachlor to heptachlor epoxide, it is likely that a large part of the toxicant would be lost in the column during cleanup.

Based on routine analysis of aldrin in rat fat, Bann, DeCino, Earle, and Sun (5) found that bioassay gave much higher results than colorimetric analysis (Table III). This led to the discovery of the change of aldrin to dieldrin in the animal body. Since the aldrin colorimetric method (23) does not give a color reaction with dieldrin, and since aldrin and dieldrin give approximately the same toxicity by the dry-film method (103), bioassay results should be much higher than those obtained by colorimetric analysis of aldrin. Such large differences in results between two types of assays would indicate the presence of a toxic metabolite.

Glasser *et al.* (46) used bioassay, colorimetric, and total chlorine methods for the detection of aldrin residues in carrots grown in aldrin-treated soil. They found that the variance in aldrin residue is due to the presence of a toxic material which gives a color reaction with the reduction-phenyl azide method. Assuming that the reduction-phenyl azide method has conclusively shown that the toxic material is dieldrin, then aldrin has been partially converted to dieldrin in either the soil or carrot tissue.

The total chlorine method was also used by Ely *et al.* (30) to study the excretion of aldrin in milk and the accumulation of aldrin in body tissues. Based on the ready change of aldrin to dieldrin (5) in animal bodies, the results obtained by the total chlorine method represent mostly dieldrin rather than aldrin. These data would agree better with bioassay results than with colorimetric analyses, because dieldrin gives approximately the same toxicity by the dry-film method (103), but is not detected by the aldrin colorimetric analysis.

Dahm, Fairchild and Robbins (21) extensively reviewed the accumulation of DDT, DDD, BHC, lindane, chlordane, aldrin, dieldrin, toxaphene and parathion in various tissues and products of several animals. Data were obtained by either bioassay or chemical analysis. Since some of the toxicants are now known to undergo metabolic changes, the meaning of some of these early results may have to be interpreted differently.

## References

1. Anderson, B. G., *Sewage Works J.*, *16*, 1156 (1944).
2. Anderson, B. G., *Science*, *102*, 539 (1945).
3. Anderson, B. G., *Trans. Am. Fisheries Soc.*, *78*, 97 (1948).
4. Angelotti, R., Fletcher, J. A., Brown, H. D., and Weiser H. H., *Proc. Am. Soc. Hort. Sci.*, *63*, 285 (1954).
5. Bann, J. M., DeCino, T. J., Earle, N. W., and Sun, Y. P., The fate of aldrin

and dieldrin in the animal body. Presented at the 128th National Am. Chem. Soc. Meeting, Sept. 1955.

6. Barnes, M. M., Carman, G. E., Ewart, W. H., and Gunther, F. A., *Advances in Chem. Ser., No. 1,* 112 (1950).
7. Bartlett, B. R., *J. Econ. Entomol., 44,* 621 (1951).
8. Bliss, C. I., *Science, 79,* 38, 409 (1934).
9. Bliss, C. I., *Ann. Appl. Biol., 22,* 307 (1935).
10. Burchfield, H. P., and Hartzell, A., *J. Econ. Entomol., 48,* 210 (1955).
11. Burchfield, H. P., Hilchey, J. D., and Storrs, E. E., *Contribs. Boyce Thompson Inst., 17,* 57 (1952).
12. Burchfield, H. P., and Storrs, E. E., *Contribs. Boyce Thompson Inst., 17,* 333 (1953).
13. Burchfield, H. P., and Storrs, E. E., *Contribs. Boyce Thompson Inst., 17,* 439 (1954).
14. Bushland, R. C., *J. Econ. Entomol., 44,* 421 (1951).
15. Callaway, S., Dirnhuber, P., and Wilson, K. M., *Nature, 170,* 843 (1952).
16. Carman, G. E., Ewart, W. H., Barnes, M. M., and Gunther, F. A., *Advances in Chem. Ser., No. 1,* 128 (1950).
17. Carter, R. H., Hubanks, P. E., Mann, H. D., Smith, F. F., Piquett, P. Q., Shaw, J. C., and Ditman, L. P., *J. Econ. Entomol., 42,* 119 (1949).
18. Carter, R. H., Nelson, R. H., and Gersdorff, W. A., *Advances in Chem. Ser., No. 1,* 271 (1950).
19. Claborn, H. V., and Wells, R. W., *Agr. Chemicals, 8,* No. 10, 28 (1952).
20. Dahm, P. A., *J. Econ. Entomol., 45,* 763 (1952).
21. Dahm, P. A., Fairchild, H. E., and Robbins, W. E., Insecticide residues in animal tissues and products. Presented at the 121st National Am. Chem. Soc. Meeting, April 1952.
22. Dahm, P. A., and Pankaskie, J. E., *J. Econ. Entomol., 42,* 987 (1949).
23. Danish, A. A., and Lidov, R. E., *Advances in Chem. Ser., No. 1,* 190 (1950).
24. Davidow, B., *J. Assoc. Offic. Agr. Chemists, 33,* 886 (1950).
25. Davidow, B., and Laug, E. P., *J. Econ. Entomol., 48,* 659 (1955).
26. Davidow, B., and Radomski, J. L., *J. Pharmacol. Exptl. Therap., 107,* 259 (1953).
27. Davidow, B., Radomski, J. L., and Ely, R. E., *Science, 118,* 383 (1953).
28. Davidow, B., and Sabatino, F. J., *J. Assoc. Offic. Agr. Chemists, 37,* 902 (1954).
29. Diephuis, F., and Dunn, C. L., *Montana Expt. Sta. Bull. 461* (1949).
30. Ely, R. E., Moore, L. A., Hubanks, P. E., Carter, R. H., and Poos, F. W., *U. S. Dept. Agr., Bur. Dairy Ind.,* BDI-INF-156 (1953).
31. Ely, R. E., and Moore, L. A., Hubanks, P. E., Carter, R. H., and Poos, F. W., *J. Dairy Sci., 38,* 669 (1955).
32. Erwin, W. R., Schiller, D., and Hoskins, W. M., *J. Agr. Food Chem., 3,* 676 (1955).
33. Fahey, J. E., *Digest of the Literature, 1925 through 1952, Relating to Sampling of Crops for Spray Residue Analysis. U. S. Dept. Agr., Mimeo* (1953).
34. Fahey, J. E., *Agr. Chemicals, 10,* No. 8, 37 (1955).
35. Fairchild, H. E., and Dahm, P. A., *J. Econ. Entomol., 48,* 141 (1955).

36. Finney, D. J., *Probit Analysis; A Statistical Treatment of the Sigmoid Response Curve*, Cambridge Univ. Press, 2nd ed., 1952.
37. Finney, D. J., *Statistical Method in Biological Assay*, Hafner Publishing Co., New York, 1952.
38. Fisher, R. W., and Smallman, B. N., *Can. Entomologist, 86*, 562 (1954).
39. Fleming, W. E., and Baker, F. E., *J. Agr. Research, 49*, 29 (1934).
40. Fleming, W. E., Coles, L. W., and Maines, W. W., *J. Econ. Entomol., 44*, 310 (1951).
41. Fleming, W. E., and Maines, W. W., *J. Econ. Entomol., 46*, 445 (1953).
42. Fleming, W. E., and Maines, W. W., *J. Econ. Entomol., 47*, 165 (1954).
43. Fleming, W. E., Maines, W. W., and Coles, L. W., *U. S. Dept. Agr., Bur. Entomol. Plant Quarantine, No. E-829* (1951).
44. Frawley, J. P., Laug, E. P., and Fitzhugh, O. G., *J. Assoc. Offic. Agr. Chemists, 35*, 741 (1952).
45. Furman, D. P., and Hoskins, W. M., *J. Econ. Entomol., 41*, 106 (1948).
46. Glasser, R. F., Blenk, R. G., Dewey, J. E., Hilton, B. D., and Weiden, M. H. J., Evidence of the presence of a toxic metabolite of aldrin in carrot. Presented at the Eastern Branch Meeting, Entomol. Soc. Amer., Nov. 1955.
47. Gunther, F. A., and Blinn, R. C., *J. Agr. Food Chem., 1*, 325 (1953).
48. Gunther, F. A., and Blinn, R. C., *Analysis of Insecticides and Acaricides*, Interscience Publishers, Inc., New York, 1955.
49. Gyrisco, G. G., Evans, W. G., Burrage, R. H., and Briant, A. M., *J. Econ. Entomol., 48*, 700 (1955).
50. Harris, D. A., *Anal. Chem., 27*, 1690 (1955).
51. Hartzell, A., *Advances in Chem. Ser., No. 1*, 99 (1950).
52. Hartzell, A., and Storrs, E. E., *Contribs. Boyce Thompson Inst., 16*, 47 (1950).
53. Hartzell, A., Storrs, E. E., and Burchfield, H. P., *Contribs. Boyce Thompson Inst., 17*, 383 (1954).
54. Heatley, N. G., *Biochem. J. (London), 41*, XIII (1947).
55. Hensil, G. S., and Gardner, L. R., *Advances in Chem. Ser., No. 1*, 102 (1950).
56. Horsfall, J. G., Heuberger, J. W., Sharvelle, E. G., and Hamilton, J. M., *Phytopathology, 30*, 545 (1940).
57. Hoskins, W. M., *J. Econ. Entomol., 42*, 966 (1949).
58. Hoskins, W. M., and Messenger, P. S., *Advances in Chem. Ser., No. 1*, 93 (1950).
59. Hoskins, W. M., Schiller, D., and Erwin, W. R., Purification of plant material and separation of insecticides for bioassay. Presented at 48th Annual Convention, Nat. Canners' Assoc., Feb. 1955.
60. Hoskins, W. M., Witt, J. M., and Erwin, W. R., *Anal. Chem., 24*, 555 (1952).
61. Jones, L. R., and Riddick, J. A., *Anal. Chem., 24*, 569 (1952).
62. Kitselman, C. H., Dahm, P. A., and Borgmann, A. R., *Am. J. Vet. Research, 11*, 378 (1950).
63. Klein, A. K., Laug, E. P., Tighe, J. F., Ramsey, L. L., Mitchell, L. C., and Kunze, F. M., *J. Assoc. Offic. Agr. Chemists, 39*, 242 (1956).
64. Kocher, C., Roth, W., and Trebaux, J., *Mitt. schweiz. entomol. Ges., 26*, 47 (1953).

65. Lamb, F. C., Development of a simplified bioassay procedure for determining insecticide residues in raw and canned products. Presented at the 48th Annual Convention, Nat. Canners' Assoc., Feb. 1955.
66. Lange, W. H., and Carlson, E. C., Residual soil insecticides for the control of wireworms affecting vegetable crops. Presented at the Entomological Club of Southern California, March 1956.
67. Laug, E. P., *J. Pharmacol. Exptl. Therap.*, *86*, 324 (1946).
68. Laug, E. P., *J. Pharmacol. Exptl. Therap.*, *93*, 277 (1948).
69. Laug, E. P., and Fitzhugh, O. G., *J. Pharmacol. Exptl. Therap.*, *87*, 18 (1946).
70. Lees, K. A., and Tootill, J. P. R., *Analyst*, *80*, 95 (1955).
71. Lees, K. A., and Tootill, J. P. R., *Analyst*, *80*, 110 (1955).
72. Lindquist, A. W., Roth, A. R., Yates, W. W., Hoffman, R. A., and Butts, J. S., *J. Econ. Entomol.*, *44*, 167 (1951).
73. March, R. B., *J. Econ. Entomol.*, *45*, 452 (1952).
74. Metcalf, R. L., and March, R. B., *J. Econ. Entomol.*, *42*, 721 (1949).
75. Metcalf, R. L., and March, R. B., *J. Econ. Entomol.*, *45*, 988 (1952).
76. Michael, A. S., Thompson, C. G., and Abramovitz, M., *Science*, *123*, 464 (1956).
77. Michael, A. S., Thompson, C. G., and Abramovitz, M., *U. S. Dept. Agr., Entomol. Research Branch*, ARS-33-15 (1956).
78. Newman, J. F., *Chemistry & Industry*, *1954*, 617.
79. Nolan, K., and Wilcoxon, F., *Agr. Chemicals*, *5*, No. 1, 53 (1950).
80. O'Donnell, A. E., Neal, M. M., Weiss, F. T., Bann, J. M., DeCino, T. J., and Lau, S. C., *J. Agr. Food Chem.*, *2*, 573 (1954).
81. O'Donnell, A. E., Johnson, H. W., Jr., and Weiss, F. T., *J. Agr. Food Chem.*, *3*, 757 (1955).
82. Olson, R. E., *The Use of the Fruit Fly, Drosophila melanogaster as a Bioassay in Detecting Minute Quantities of Benzene Hexachloride in Plant Tissue*, Cornell Univ., Ph.D. Thesis, 1954.
83. Pagan, C., *J. Econ. Entomol.*, *41*, 942 (1949).
84. Pagan, C., and Hageman, R. H., *Science*, *112*, 222 (1950).
85. Pankaskie, J. E., and Sun, Y. P., *III$^e$ Congr. intern. phytopharmacie*, Paris, 52–55 (1950).
86. Patterson, W. I., and Lehman, A. J., *Assoc. Food & Drug Officials U. S., Quart. Bull.*, *17*, 3 (1953).
87. Pfadt, R. E., and Ryff, J. F., *J. Econ. Entomol.*, *48*, 195 (1955).
88. Pfaff, W., *Z. Pflanzenkrankh. u. Pflanzenschutz*, *62*, 361 (1955).
89. Pratt, R., and Dufrenoy, J., *Antibiotics*, 2nd ed., Lippincott, Philadelphia, 1953.
90. Radomski, J. L., and Davidow, B., *J. Pharmacol. Exptl. Therap.*, *107*, 266 (1953).
91. Rideal, S., and Walker, J. F. A., *J. Roy. Sanit. Inst. (London)* (1903) (cited by Young and Cooper, 1917).
92. Robbins, W. E., and Dahm, P. A., *J. Agr. Food Chem.*, *3*, 500 (1955).
93. Shell Development Co., Extraction of Pesticide Residues from Crops and Animal Products, *Shell Method Series* 551/55 (1955).
94. Shell Development Co., unpublished data.

95. Simmons, S. W., *U. S. Pub. Health Service, Suppl. Public Health Repts., No. 186*, 3–20 (1945).
96. Stansbury, R. E., and Dahm, P. A., *J. Econ. Entomol., 44*, 45 (1951).
97. Starnes, O., *J. Econ. Entomol., 43*, 338 (1950).
98. Sun, J. Y. Tung, and Sun, Y. P., *J. Econ. Entomol., 46*, 927 (1953).
99. Sun, Y. P., *Minn. Agr. Expt. Sta., Tech. Bull. 177* (1947).
100. Sun, Y. P., *J. Econ. Entomol., 43*, 45 (1950).
101. Sun, Y. P., and Pankaskie, J. E., *J. Econ. Entomol., 47*, 180 (1954).
102. Sun, Y. P., Pankaskie, J. E., and Sun, J. Y. Tung, *Some important factors affecting microbioassay of insecticide residues.* Presented at the 63rd Annual Meeting, Amer. Assoc. Econ. Entomol., Dec. 1951.
103. Sun, Y. P., and Sun, J. Y. Tung, *J. Econ. Entomol., 45*, 26 (1952).
104. Terricre, L. C., and Ingalsbe, D. W., *J. Econ. Entomol., 46*, 751 (1953).
105. Terriere, L. C., and Kiigemagi, U., Simplified bioassay for insecticide residues. Presented at the 129th National Am. Chem. Soc. Meeting, April 1956.
106. Treon, J. F., Cleveland, F. P., and Cappel, J., *J. Agr. Food Chem., 3*, 842 (1955).
107. Tressler, C. J., and Jenkins, T. H., Insecticide residue determination using the Hoskins purification procedure and Laug housefly bioassay method. Presented at the 48th Annual Convention, Nat. Canners' Assoc., Feb. 1955.
108. Ward, J., and Burt, P. E., *Bull. Entomol. Research, 46*, 849 (1956).
109. Wasserburger, H. J., *Pharmazie, 7*, 731 (1952).
110. Wilcoxon, F., *J. Econ. Entomol., 39*, 269 (1946).
111. Wilcoxon, F., and McCallan, S. E. A., *Contribs. Boyce Thompson Inst., 10*, 329 (1939).
112. Young, H. C., and Cooper, E. H., *Mich. Acad. Sci. Rept., 19*, 221 (1917).

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